ANTIGENIC COMPOSITION OF FIVE STRAINS OF HERPES SIMPLEX VIRUS TYPE ONE THAT CAUSE CHARACTERISTIC OCULAR DISEASE

By

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A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1980

ACKNOWLEDGMENTS

The author expresses appreciation to Drs. Ysolina M. Centifanto, Herbert E. Kaufman, Kenneth I. Berns, George E. Gifford, and Donna H. Duckworth for their suggestions while serving on her supervisory committee. She is especially grateful to Dr. Ysolina M. Centifanto for her constant intellectual stimulation, guidance, advice, support, and patience during the course of this research and preparation of this dissertation.

The author expresses appreciation to the Departments of Ophthalmology at the University of Florida and Louisiana State University Medical Center for the financial assistance provided. She is grateful to Theresa Moorehead, the members of the Editorial Office and the Department of Photography at the Louisiana State University Eye Center, and the Department of Learning Resources at the Louisiana State University Medical Center for their technical assistance in the preparation of this dissertation. Special thanks go to the members of Dr. Centifanto's laboratories at the University of Florida and Louisiana State University Medical Center for their support and assistance.

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June 1980

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Major Department: Immunology and Medical Microbiology

The antigenic composition of five strains of herpes simplex virus (HSV) that cause characteristic ocular disease of varying severity in New Zealand white rabbits has been studied by crossed immunoelectrophoresis. Antiserum to each strain was raised by intramuscular immunization and boosting by infection in the eyes of rabbits. The immunoglobulin fraction was obtained by ammonium sulfate precipitation. Crossed immunoelectrophoresis using homologous antibodies showed that the Shealy and RE strains had thirteen virus-specific antigens, the McKrae strain twelve, the F strain eleven, and the CGA-3 strain eight. Experiments using heterologous antibodies showed that three antigens were common to all five strains, ten were shared by two or more strains, and ten were characteristic of one strain only. The data indicated that there was a correlation between the antigenic composition of the five strains and the kind and severity

of ocular disease produced. As the severity of disease increased, the number of virus-specific antigens increased. Similarities in antigenic composition among the strains also correlated with the kind of ocular disease produced. It is hoped that the correlation of the antigenic composition with other biological and biochemical properties may prove helpful in understanding the pathogenesis of HSV in ocular disease.

INTRODUCTION

Herpes simplex virus (HSV) can infect almost every organ of the body and is involved in many diseases of man including, perhaps, some forms of cancer. The virus has a predilection for the skin, genitals, and eye, and infections of these tissues are common. Less often HSV infects sites such as visceral organs and the central nervous system and may result in fatal disease (43). Human isolates of HSV are divided into two serotypes, type 1 and type 2, based on antigenic and biological differences (53). Generally, it is said that strains of HSV-1 are associated with infections above the waist and strains of HSV-2 with infections below the waist (53).

Ocular infections caused by HSV are very troublesome. In the United States, the most common and significant virus infection of the cornea is caused by HSV (29). Isolates from eye lesions are usually type 1, and both primary and recurrent infections can occur (53). Ocular infections by HSV result in a range of disease from mild conjunctivitis and dendritic keratitis to more severe disciform edema and necrotizing stromal keratitis. The latter may result in blindness (30).

HSV-induced ocular disease is an excellent model for the study of causes of variation in the disease states produced by HSV. For many years it was thought that differences in the hosts such as genetic (HLA type), hormonal, and immunologic factors were responsible for the varying forms of eye disease, and much work was done to try to confirm

this. Results from these studies have been disappointing. Recently, differences in biological and biochemical properties of the infecting viruses have been implicated as a factor in the variation in eye disease, and investigators are now beginning to study these strain differences in relation to disease.

Centifanto and associates (8,9) are presently investigating differences in HSV strains by comparing their cytopathic effects in vitro, plaque morphology (social behavior), sensitivity to antiviral drugs, and their polypeptide composition with the kind, severity, and virulence of disease produced in the rabbit cornea by these strains. In the work to be presented here, the antigenic composition of five strains of HSV type 1 was determined and correlated with the kind and severity of eye disease that they caused. It is hoped that the correlation of antigenic composition with other strain differences and with the ocular disease pattern will help in the understanding of the pathogenesis of HSV.

Virus Structure and Chemical Composition

HSV type 1 is a member of the Herpesviridae (22). The morphology of the herpesviruses is the basis for their classification in this family (80). Their virions are seen, by electron microscopic examination of negatively stained preparations, to be divided into three parts: the core, the capsid, and the outer membrane or envelope. The core, 77.5 nm in diameter, contains the double-stranded DNA genome. The capsid encloses the core, is 105 nm in diameter, is spherical or hexagonal, and is composed of 162 capsomeres (150 hexagonal and 12 pentagonal) arranged to give icosahedral symmetry. The capsomeres are

approximately 100 Å in diameter with a characteristic axial hole about 40 Å in diameter. The nucleocapsid is surrounded by an outer membrane or envelope, and the enveloped nuleocapsid varies in size from 150 to 200 nm.

The major components of the virion are DNA, proteins, glycoproteins, polyamines, and lipids (58). The buoyant density of the complete virion is 1.27 to 1.85 g/cm^3 determined by equilibrium centrifugation in cesium chloride (CsCl) density gradients (68,69).

Virus DNA

The DNA found in an HSV virion is a double-stranded, linear DNA molecule (4,32). Kieff et al. (32) established the molecular weight of the DNA of HSV-1 as (99 ± 5) x 10^6 daltons, using its rate of co-sedimentation with nonglycosylated T4 DNA. These reults are compatible with the measure of (95 ± 1) x 10^6 determined by kinetic complexity (13), with the measure of 95.7 x 10^6 established by measuring the contour length of DNA (58), and with other determinations (14,82). The guanine plus cytosine content of HSV-1 is 67 moles percent, as demonstrated by equilibrium banding in analytical CsCl density gradients and thermal denaturation (melting) temperature (32).

Virus Proteins

Based on kinetic, immunologic, and genetic criteria, approximately 48 virus-specific polypeptides ranging in molecular weight from 15,000 to 280,000 are separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of HSV-1 infected cells (24,25). Twenty-four of these 48 polypeptides are found in the virion and are

classified as structural (66); fifteen are nonstructural, and 9 are unclassified polypeptides (24).

Naked nucleocapsids contain only four to eight polypeptides. This suggests that most of the proteins and glycoproteins of the virion are obtained during envelopment (67). One major protein of molecular weight 140,000 to 160,000 daltons that is found in the nucleocapsid of many herpesviruses is probably the major hexon protein. Since empty capsids of HSV lack two of the nucleocapsid proteins, a role is implied for these proteins in the packaging of the genome.

Virus Envelope

The envelopment of herpesviruses occurs at the inner nuclear membrane (67). Although the virion buds through the membrane, the virion envelope lacks most of the normal host cell proteins. This occurrence implies a rearrangement of the membrane components (67). In all herpesviruses studied, there are ten to fifteen nonglycosylated proteins and approximately six glycoproteins which migrate from their sites of synthesis in the cytoplasm to the inner nuclear membrane and are acquired by the virus during envelopment. These virus-specific glycoproteins are integral membrane proteins with their carbohydrate moieties on the outer surface of the virion. The virus-specific nonglycosylated proteins are important in the envelopment of the nucleocapsid and are located between the nucleocapsid surface and the inner surface of the lipid bilayer of the envelope (67).

The virion envelope, with its associated glycoproteins, plays a pivotal role in the entrance of the nucleocapsid into a cell during the initiation of infection (67). This conclusion is based on studies

indicating that naked nucleocapsids are not infectious, that neutralizing antibodies are made to envelope components, and that virion envelopes lacking the glycoprotein VP7(B_2) exhibit low infectivity. The envelope of the virion apparently fuses with the host cell membrane, and the nucleocapsid is released into the cytoplasm, thereby initiating the infection (42).

Social Behavior of Virus Infected Cells

Studies of the "social behavior" of HSV infected cells demonstrate that the virus-specific structural glycoproteins are also important in the interaction of infected cells with each other. The normal (wild type or *syn*$^+$) expression of this interaction is the clumping of rounded cells (60). The variant expression (*syn*) is polykaryocytosis, i.e. giant cell formation into syncitium (60). Since either clumping or fusion requires the interaction of the plasma membrane of adjacent cells, Roizman (56,57) proposed that changes in the glycoproteins affect the plasma membrane of infected cells and that this results in changes in immunological specificity and alteration in social behavior. Later studies confirm this hypothesis (45,55,59).

The finding that viruses that cause different social behavior express different membrane glycoproteins identified on SDS-PAGE (31), as well as studies with the mutant strains, HSV-1(MP) and HSV-1(13VR4), which cause polykaryocytosis and do not accumulate VP8(C_2) (37,60), demonstrate the role of membrane glycoproteins in social behavior. In confirmatory studies on the relationship between the glycoproteins and the interactions of HSV infected cells, Ruyechan *et al*. (60) mapped the location on the HSV DNA of (a) three mutations at three different loci

that alter social behavior from wild type rounded cells in clumps to polykaryocytosis, (b) one mutation which accumulates one of the major structural glycoproteins, [VP8(C_2)], and (c) the sequences for the four major structural glycoproteins, VP8(C_2), VP7(B_2), VP8.5(A), and VP19E(D_2).

Two of the three loci that determine the social behavior of infected cells, syn 1 and syn 2, do not map at the same location as the structural genes encoding glycoproteins VP8(C_2), VP7(B_2), VP8.5 (A) and VP19E(D_2) (60). These mutations could possibly be in minor glycosylated proteins which are not yet characterized, nonglycosylated proteins, or enzymes which affect membrane proteins. The third locus, syn 3, is found within the boundaries of the template for glycoproteins VP8.5(A) and VP7(B_2). Although it is not known whether syn 3 is found within the templates specifying these glycoproteins or other unmapped proteins, glycoprotein VP7(B_2) does play a significant role in cell fusion (37,61). The Cr locus, which determines the accumulation of glycoprotein VP8(C_2) maps to the right of the structural gene for VP8(C_2). Loci syn 1 and syn 2 are found at or near this Cr locus but can be segregated from it. Since both spontaneous HSV-1(MP) and mutagen-induced HSV-1(13VB4) mutants show the Cr^- syn phenotype, perhaps there is a relationship between these loci. It is possible that the template specifying VP8(C_2), the Cr locus, and the syn 2 locus may be within a transcriptional unit or in overlapping templates.

Virus Glycoproteins

Virions of HSV-1 exhibit five antigenically distinct glycoproteins called VP8(C_2), VP7(B_2), VP8.5(A), VP18(D_2), (67) and E_2 (3).

Virally-induced glycoproteins are incorporated into the cellular membranes of infected cells (20,21). These glycoproteins in the cell membrane cannot be differentiated from the structural virion glycoproteins on the basis of their number or electrophoretic mobility (26,64). The structural glycoproteins which are exposed on the virion surface are responsible for virus adsorption and penetration and are targets of neutralizing antibodies (67).

Studies with mutants have indicated the specific function for several of the glycoproteins. Glycoprotein VP8(C_2) is not important in viral morphogenesis or adsorption as shown by one spontaneous mutant, HSV-1(MP), which yields infectious virions but does not make VP8(C_2). This glycoprotein is associated with the suppression of HSV-induced cell fusion as shown by the mutant HSV-1(MP) which causes polykaryocytosis and other studies which indicate that mutants that do not produce this glycoprotein also have this effect (37). Studies using the temperature-sensitive mutant HSV-1[HFEM]tsB5 show that glycoprotein VP7(B_2) is not required for viral morphogenesis or virus adsorption but is necessary for penetration (67). At the nonpermissive temperature, 39°C, this mutant virus does not produce functional VP7(B_2) and yields noninfectious virions which lack this glycoprotein. These virions adsorb to cells but do not start infection; after addition of polyethylene glycol, which promotes membrane fusion, the infectivity of this mutant increased (61). Other studies indicate that this glycoprotein plays a significant role in HSV-induced cell fusion presumably by promoting fusion between the envelope of the virions and the membrane of the host cell (37,61).

Glycoprotein E_2 binds to the Fc part of the immunoglobulin G molecule, and anti-E_2 has neutralizing activity in a complement-dependent reaction (Spear, personal communication). The specific functions of VP18(D_2) and VP8.5(A) are not known.

In summary, glycoprotein VP8(C_2) is not required for infectivity, virus morphogenesis, or penetration, and mutants that do not produce VP8(C_2) usually cause polykaryocytosis. VP7(B_2) is required for infectivity but not for virus morphogenesis or adsorption; it is necessary for penetration and plays a role in HSV-induced cell fusion. Glycoprotein E_2 binds to the Fc portion of the immuoglobulin G molecule. The roles of glycoproteins VP8.5(A) and VP18(D_2) are not known. Glycoprotein VP8.5(A) is related to glycoprotein VP7(B_2) and may be a separate form or breakdown product of VP7(B_2). Three of the glycoproteins [VP7(B_2), VP18(D_2), E_2] elicit the production of neutralizing antibodies in immunized New Zealand white rabbits.

Virus Antigens

As seen from the preceding discussion, virus infection results in the formation of many new proteins and glycoproteins. These may all serve as antigens. Several factors determine if an antigen is biologically significant: its location within the cell, whether or not it is a structural or nonstructural protein, its exposure on the surface of the virion or the infected cell membrane, its interaction with host cell components, its immunogenicity, and its role in the immunopathological process (7). Many serological techniques including neutralization, immunofluorescence, immunoferritin staining, immunoperoxidase staining, immune agglutination of virus particles,

complement fixation, immunodiffusion, crossed immunoelectrophoresis, and immunocytolysis are used in characterizing antigens in HSV infected cells both *in vitro* and *in vivo* (16).

Using one of these methods, agar gel immunodiffusion, Watson *et al.* (81) demonstrated 12 virus-specific antigens of HSV-1 infected cells, and Honess *et al.* (23) demonstrated 11 and showed that six of these antigens were type specific and five type common. This study demonstrated that seven of the 11 were structural antigens (three type specific and four type common) and that two of these structural antigens were involved in type specific neutralization and one in type common neutralization. One of the nonstructural type specific antigens was thymidine kinase, which was not glycosylated (27). The functions of the other antigens were unknown.

Using other techniques, Cohen *et al.* (10) described the CP-1 antigen, which is a type common, glycosylated antigen that elicits the production of neutralizing antibody and is detected in soluble form in infected cells, in detergent extracts of infected cell membranes, and in the envelope of the HSV virion. Cohen has correlated his results with those of other laboratories, stating that CP-1 is possibly related to Spear's glycoprotein VP18(D_2) (64), VP17, 18, 19E of Heine *et al.* (19), Norrild and Vestergaard's Ag8 (49), and 8/9 of Powell *et al.* (52).

In recent studies using crossed immunoelectrophoresis (cIEP), the antigens of HSV-1 and HSV-2 infected cells have been characterized by their electrophoretic mobility in antibody-containing agarose gels (71). The membranes of HSV infected cells have been solubilized with

the nonionic detergent Triton X-100, which preserves the native macromolecular protein conformation and the immunologic reactivity of the antigens (5). Eleven antigens were originally identified by this method (71). Extensive studies were done to further characterize these antigens including determination of the immunological significance with respect to neutralization and immunocytolysis, polypeptide composition, and location and time of appearance postinfection (46-50, 71-78).

One of the most important antigens is antigen 11 (Ag11). This antigen is glycosylated (47,49,74) and membrane-bound (73) and has both type common and type-1 specific antigenic sites (71,76). It has also been shown to be a virion protein by Commassie brilliant blue (CBB) staining of cIEP gels and has been shown on the surface of infected cells 5 h postinfection (46). Antigen 11 eluted from the immunoprecipitates in antibody-containing gels of cIEP is composed of three polypeptides as seen on SDS-PAGE, including glycoproteins VP8.5(A) and VP7(B_2) (48,49). When lactoperoxidase-catalyzed iodination was used, only one polypeptide of the Ag11 complex of molecular weight 127 x 10^{-3} was found on the surface of 18 h infected cells (46), and this polypeptide was shown to be glycoprotein VP7(B_2) (47,49). Using cIEP and rocket electrophoresis, Norrild et al. (47) demonstrated that this antigen is a common antigen of cells infected with 4 herpesviruses: HSV-1, HSV-2, bovine mammillitis virus, and B virus. Although the composition of Ag11 differed in the four viruses, glycoprotein VP7(B_2) was demonstrated in all four herpesviruses, indicating that the common antigenic site is on the glycoprotein.

Antigen 8 (Ag8) also has both type common and type-1 specific antigenic sites (71,72,76). It is a glycosylated, membrane-bound antigen found on the virion and on the surface of infected cells at 8 h postinfection (46,47,49,71-74,76). This antigen is composed of several polypeptides including various forms of glycoprotein VP18(D_2) (49,50). Three of these glycopolypeptides of molecular weight 80 x 10^{-3}, 73 x 10^{-3}, and 64 x 10^{-3} are exposed on the surface of 18 h infected cells (46). Recent immunoelectron microscopy studies using peroxidase-antiperoxidase (unlabeled antibody-enzyme method) and monospecific anti-Ag8 demonstrated that Ag8 is localized at both the nuclear and plasma membranes of HSV infected cells and is detected both early (2 h postinfection) and late (19 h postinfection) (17). These studies support an earlier finding that showed that most of the virus-specific proteins are present from early to late stages of infection, but in different amounts (24).

Monoprecipitin antisera to Ag11 and Ag8 have high neutralizing antibody titers against both HSV-1 and HSV-2 (78) and mediate immunocytolysis in the presence of complement (antibody-dependent complement-mediated cytotoxicity) or peripheral blood mononuclear cells (antibody-dependent cell-mediated cytotoxicity or ADCC) (48). High antibody titers against these two antigens were found in 100 human sera tested and these titers correlated with HSV neutralizing antibody titers (72).

A third type common antigen is antigen 3 (Ag3) which is water-soluble, nonglycosylated and composed of several polypeptides (49,71,73). This antigen is not detected on purified HSV-1 virion

preparations by cIEP and is not exposed on the surface of infected cells (46). Vestergaard (72) speculates that Ag3 may be a major capsid antigen, but the nature of this antigen is presently unknown. Antibodies to Ag3 have also been demonstrated in human sera (72), and rabbits produce antibodies against Ag3 rapidly (73); however, the immunological significance of these antibodies is presently unknown.

An interesting antigen which is HSV type 1-specific is antigen 6 (Ag6). This antigen is a virion protein that is glycosylated, membrane-bound, and located on the surface of infected cells between 8 and 18 h postinfection (46,71,74). The major polypeptide is glycoprotein VP8(C_2) as seen on SDS-PAGE (48,77). This is confirmed by studies showing that this antigen is not detected in cIEP of HSV-1(MP) infected cells that lack glycoprotein VP8(C_2). Furthermore, monoprecipitin antiserum to Ag6 did not react in the ADCC reaction with either Chang liver cells or HEp-2 cells infected with HSV-1(MP) (48).

When lactoperoxidase-catalyzed iodination of HSV infected cells was used, Ag6 of HSV-1(VR3) infected Chang liver cells was shown on the surface of 8 h infected cells (48) in contrast to the same antigen of HSV-1(F) infected HEp-2 cells which was shown on the surface only late in infection (8 to 18 h postinfection) (46). Although two different strains of virus were used, the difference in the surface expression of this antigen appears to be host cell dependent (48). In addition, anti-Ag6 resulted in a lower ADCC reaction in HEp-2 infected cells than in Chang liver infected cells (48). These studies agree with the

demonstration that mutations in the syn^1 and syn^2 loci specifying social behavior of HSV infected cells are host cell dependent (60).

Monoprecipitin antisera to Ag6 has both neutralizing and immunocytolytic activities against HSV-1 infected cells (48,78). Antibodies to this antigen were shown in human sera, and the titers correlated better with HSV-1 than HSV-2 neutralizing titers (72).

Another antigen which is possibly a HSV type 1-specific antigen is antigen 7 (Ag7) (71). It is a virion protein which is membrane-bound, glycosylated (76; Norrild, personal communication) and exposed on the surface of HSV-1 infected cells between 8 and 18 h postinfection (46). Another antigen which has recently been identified on cIEP is antigen 5 (Ag5), which is composed of the major capsid protein, ICP-5 (19,48).

Two antigens which are HSV type 2-specific are antigen 4 (Ag4) and antigen 9 (Ag9) (73,76). Both of these antigens are membrane-bound and glycosylated (71,74,76). Anti-Ag4 and anti-Ag9 both neutralize HSV-2 to a greater extent than they do HSV-1 (78). Also, antibodies to these two antigens were demonstrated in human sera and the titers correlated better with HSV-2 neutralizing titers than HSV-1 titers (72).

In summary, antigens of HSV infected cells have been characterized by several serological techniques including immunodiffusion and crossed immunoelectrophoresis. The immunological significance of these antigens has been demonstrated by neutralization and immunocytolysis. When cIEP in agarose gel was used, seven antigens were characterized in various strains of HSV-1 (3,3A,5,6,7,8,11) and eight antigens in various strains of HSV-2 (1,2,3,4,8,9,10,11). Antigens 3, 8, and 11 are type common; Ag6 and possibly Ag7 are type 1-specific, and Ag4 and

Ag 9 are type 2-specific. Antigens 6, 8, and 11 are virion proteins which are glycosylated, membrane-bound, and found on the surface of infected cells. Antigen 6 is composed of glycoprotein VP8(C_2); Ag8 of glycoprotein VP18(D_2), and Ag11 of glycoproteins VP8.5(A) and VP7(B_2). Antigen 7 is a glycosylated, membrane-bound, virion protein located on the surface of infected cells. Antigens 4 and 9 are glycosylated and membrane-bound. Antigens 1, 3 and 5 are not glycosylated. Monoprecipitin antisera to antigens 4,6,8,9 and 11 have neutralizing activity and anti-6, -8 and -11 can also mediate immunocytolysis. Vestergaard (72) proposed that in human sera the serological cross-reactivity of HSV-1 and HSV-2 is due to antibodies to Ag8 and Ag11, that antigens 6, 8 and 11 are responsible for the production of antibodies to HSV-1, and that antigens 4 and 9 elicit antibodies to HSV-2.

Virus and Ocular Disease

Antigens on the HSV virion and on the surface of infected cells play an important role in HSV-induced ocular disease. Infections by HSV cause both a humoral and a cellular immune response in which a variety of entities can interact with these antigens. It is also significant that HSV can infect cells involved in the immune response, such as T and B lymphocytes, macrophages, and polymorphonuclear leukocytes (43).

Varying forms of ocular disease are produced by HSV, and the different disease states have been reviewed by Kaufman (30), Metcalf and Kaufman (40), Metcalf *et al.* (41), Rawls (53) and Nahmias and Norrild (43). In acute conjunctivitis there are ulcers on the

conjunctiva and vesicle formation on the lid margin and skin. This primary infection may be limited to the conjunctiva, or the cornea may also be involved. The virus replicates in the epithelial layer of the cornea in epithelial keratitis, resulting in characteristic dendritic ulceration; punctate erosions or small dendritic ulcers may progress to larger, geographic defects. The disease is often mild, localized, and self-limiting and can be controlled by topical antiviral drugs. After a primary infection in which infectious virus can be recovered, the virus persists in a latent form in the trigeminal or other ganglia (11, 44,70). Infectious virus is not detected in the ganglia but can be demonstrated after explanting the ganglia or cocultivation with rabbit kidney cells (11,44,70; Centifanto, personal communication). This latent form of the virus is the source of possible recurrences and in recurrent keratitis, the virus is reactivated and causes deep, unbranched, marginal ulcers and large areas of erosion that can result in irreversible corneal scarring and opacification.

A more serious sequela of epithelial keratitis is the development of deep stromal disease with disciform edema (30,41,83). The milder form includes two or three months of localized edema and opacification which then heals, leaving little or no scarring. In some patients, a more severe form of the necrotizing keratitis develops with dense, cheesy opacities in the corneal stroma, corneal vascularization, and necrosis of stromal collagen fibers. This severe form of the disease results in permanent scarring and blindness.

In the rabbit cornea, different strains of HSV have been shown to produce different disease patterns, ranging from mild epithelial

keratitis to severe stromal necrotizing keratitis (63,79), and these varying disease states show many similarities to human infections (41). Virus replication occurs in epithelial keratitis, and HSV antigens have been demonstrated within the nucleus, on nuclear membranes, and on membranes of epithelial cells (39). When the disease progresses to necrotizing keratitis, viral particles are not found in the stroma; however, virus antigens are found on the surface of stromal keratocytes. Several studies indicate that viral replication is important in initiating, but not in maintaining, the disease state (38,62). In stromal keratitis an initial inflammatory response of polymorphonuclear leukocytes and macrophages is followed by lymphocytes and plasma cells (40,41), and these lymphocytes are often found in close association with the degenerating stromal keratocytes (40). The histology and pathology of stromal keratitis demonstrated in the rabbit model and the fact that disciform edema in humans is treated with corticosteroids (29,30) both indicate that immunopathogenesis has a role in the maintenance of the disease state.

The variation in ocular disease is dependent upon the differences in the host response to the infecting virus and the properties of that strain, and both have been studied for their effect on the type of eye disease caused by HSV. Since antigens have been shown to be important in infectivity, immune response, and disease, the antigenic composition of five strains of HSV type 1 that cause varying forms of ocular disease has been determined in this study by cIEP in agarose gels. Crossed immunoelectrophoresis using homologous antiserum demonstrated the virus-specific antigens of each strain. The similarities among

these strains were shown using heterologous antisera. Since glycoproteins are involved in disease, neutralization, and kind of cytopathic effect, the glycosylated antigen pattern was also determined. The antigenic composition of these five strains was found to be related to the severity and kind of eye disease they produced. It is hoped that this correlation will help in the understanding of the pathogenesis of HSV-induced ocular disease.

MATERIALS AND METHODS

Virus Strains and Cell Culture

Five strains of herpes simplex virus type 1 were used. The Shealy strain was obtained from Dr. Andre Nahmias (Emory University). The McKrae strain was isolated in Dr. Ysolina M. Centifanto's laboratory from a patient of Dr. Herbert E. Kaufman. The F strain was a gift from Dr. Bernard Roizman (University of Chicago). The RE strain was obtained from Dr. Chandler R. Dawson (Francis I. Procter Eye Institute, San Francisco). The CGA-3 strain was a gift from Dr. R. F. McNair Scott (University of Pennsylvania).

To obtain stock virus, Vero or HEp-2 cells were grown to confluency in 32 oz tissue culture flasks using complete growth medium composed of Basal Medium Eagle [BME (Microbiological Associates, Walkersville, Maryland)] or Minimum Essential Medium (Eagle) [MEM (Grand Island Biological Company, Grand Island, New York)], 10% heat-inactivated fetal calf serum [FCS (Microbiological Associates)], 10% sodium bicarbonate (7.5% solution) [$NaHCO_3$ (Flow Laboratories, Rockville, Maryland)], 1% glutamine (Grand Island Biological Company), penicillin-streptomycin mixture (Microbiological Associates) and fungizone (E. R. Squibb and Sons, Incorporated, Princeton, New Jersey). Virus was seeded on cultures and adsorbed for 1 h at 37°C. Complete maintenance medium, including the same components as growth medium with 2% $NaHCO_3$ and 2% FCS, was added. After 24 to 48 h of infection at 37°C, infected cells were frozen, thawed, dispensed, and stored at -76°C.

To grow virus stocks of very high titers, HEp-2 cells were grown to confluency in glass roller bottles (Bellco Glass, Inc., Vineland, New Jersey) with MEM, 5% non-heat-inactivated FCS, 1% $NaHCO_3$, 1% glutamine, and antibiotics. The cell layer was washed extensively with phosphate-buffered saline (PBS), pH 7.2. Stock virus was added in 5 ml volume and adsorbed for 1 h at 37°C. Medium 199 (Grand Island Biological Company) with 1% non-heat-inactivated FCS dialyzed in $NaHCO_3$, antibiotics and fungizone were added, and cells were incubated at 37°C. After 24 to 48 h of infection, the medium was decanted, and the cells were washed extensively with PBS (three to five times), scraped, pelleted by centrifugation, washed with PBS by resuspension and sedimentation, and frozen at -76°C.

To grow virus for radioactively labeled antigen for immunoelectrophoresis, HEp-2 cells were grown to confluency in 150 cm^2 tissue culture flasks (Corning Glass Works, Corning, New York) with complete growth medium. Cells were washed extensively with PBS, virus grown in roller bottles (as described above) was adsorbed for 1 h, and medium containing the radioactively labeled components was added. To radioactively label proteins, Hanks Balanced Salt Solution [HBSS (Microbiological Associates)] with 1% MEM vitamin solution (Grand Island Biological Company), 2% non-essential amino acids (one tenth the normal concentration) (Flow Laboratories), 1% $NaHCO_3$, 1% glutamine, 1 uCi/ml each ^{14}C-leucine, -isoleucine, -valine (New England Nuclear, Boston, Massachusettes) to equal 15 uCi of each amino acid, antibiotics and fungizone were added. To radioactively label glycoproteins, Medium 199 with 1% non-heat-inactivated FCS dialyzed in

$NaHCO_3$, antibiotics, fungizone, and 2 uCi/ml ^{14}C-glucosamine hydrochloride (New England Nuclear) to equal 40 uCi were added. After 24 to 48 h of infection, the cells were scraped, pelleted by centrifugation, washed with PBS three times, and frozen at -76°C.

To grow virus for immunogen, cultures of rabbit kidney-13 [RK-13 (Flow Laboratories)] were grown to confluency in 32 oz tissue culture flasks using MEM complete growth medium. The cultures were inoculated with virus which had been passaged two times (Vero, RK-13) without FCS. After 1 h of adsorption, BME with 10% NCTC 135 (Grand Island Biological Company), 1% $NaHCO_3$, 1% glutamine, 1% non-essential amino acids, antibiotics, and fungizone were added. After 24 to 48 h of infection, the cells were frozen, thawed, dispensed, and stored at -76°C.

Preparation of Antigen

To prepare antigens for immunoelectrophoresis, the procedure of Norrild and Vestergaard was followed (49,71; Norrild, personal communication; Vestergaard, personal communication). To obtain radioactively labeled infected cells, 1.0×10^7 HEp-2 cells were infected with each of the five viruses at multiplicities of infection of ten or more plaque-forming units/cell (PFU/cell). Virus was adsorbed for 1 h. To radioactively label proteins, medium containing amino acids at one tenth the normal concentration, supplemented with ^{14}C-leucine, -isoleucine, -valine, was added. To radioactively label glycoproteins, medium containing ^{14}C-glucosamine hydrochloride was added. After 24 to 48 h of infection, cells were scraped and harvested by centrifugation; the pellet was washed extensively with PBS to remove components of the medium. The protein

was solubilized in 0.0076 M tris(hydroxymethyl)aminomethane (Tris) - 0.02 M glycine containing 5% (vol/vol) Triton X-100 (octyl phenoxy polyethoxyethanol) (Sigma Chemical Company, St. Louis, Missouri) in four times the volume of the wet cell pellet. The pellet was disrupted by sonic treatment at 20,000 cycles/sec four times for 1 min at 10 min intervals. The mixture was centrifuged at 100,000 x g for 1 h, and the supernatant fluid containing the antigen was stored at -76°C. HEp-2 cells were mock infected and labeled, and antigen was prepared. Antigens with radioactively labeled proteins contained the following counts per minute, cpm/0.1 ml: Shealy- 349,528; McKrae- 69,220; F- 63,094; RE- 280,776; CGA-3- 148,204; HEp-2 mock infected- 404,070. Antigens with radioactively labeled glucosamine contained the following cpm/0.1 ml: Shealy- 128,102; McKrae- 116,814; F- 107,667; RE- 132,636; CGA-3- 243,084; and HEp-2 mock infected- 163,821.

Preparation of Antisera

Rabbit kidney-13 cells were infected with each of the five viruses passed twice without FCS. After 1 h of adsorption, BME with no FCS was added to each infected culture. After 24 to 48 h, the infected cells were frozen at -76°C. The infected cells were thawed and used as the immunogen. One milliliter of a 1:1 mixture of immunogen to Freund's Complete Adjuvant (Cappel Laboratories, Incorporated, Cochranville, Pennsylvania) was injected intramuscularly (i.m.) into each of the hind legs of a New Zealand white rabbit. Four rabbits were immunized per virus. Each rabbit was immunized weekly for three weeks, boosted by infection in the eye ten days later, and bled 6, 10, 17, and 20 days postinfection. Sera from the four rabbits immunized with the

same virus were pooled from each of the four separate bleeding dates. All five viruses were neutralized by the homologous antisera from the first and last bleeding dates. Therefore, the sera from all the bleeding dates were pooled for each antiserum.

The procedure of Harboe and Ingild (18) was adapted to obtain an immunoglobulin-rich fraction of antisera. An aliquot of the pooled serum was precipitated by ammonium sulfate overnight at 4°C. The mixture was centrifuged at 4000 rpm for 30 min, and the pellet was resuspended in the amount of deionized water approximately equal to one fifth the original volume. The mixture was dialyzed against deionized water overnight at 4°C and dialyzed against acetate buffer (0.05 M sodium acetate - 0.022 M acetic acid), pH 5.0, overnight. The antiserum was centrifuged at 10,000 rpm for 10 min to remove the lipid. The supernatant fluid was dialyzed against PBS overnight. The immunoglobulin fraction was tested against various antigens for immunoelectrophoresis by Ouchterlony agar diffusion and was used in crossed immunoelectrophoresis.

Crossed Immunoelectrophoresis

The procedure for crossed immunoelectrophoresis was modified from Norrild and Vestergaard (49; Norrild, personal communication). For electrophoresis in the first dimension, 15 ml of 1% agarose made from agarose immunoelectrophoresis tablets (Bio-Rad Laboratories, Richmond, California) containing 1% Triton X-100 was cast on a 90 mm by 110 mm glass plate. Four wells 4 mm in diameter were cut in the agarose approximately 20 mm from one edge of the plate. The antigen was applied to each well in 5, 10, or 15 ul volumes. If more than 15 ul

was necessary, either antigen was added in 15 ul aliquots and the plates placed in a moist chamber at 37°C for diffusion into the agarose, or more than one 4 mm well was cut and 15 ul of antigen added. Five microliters of 0.05% phenol red in 0.0076 M Tris - 0.02 M glycine containing 5% Triton X-100 was added to each well as a marker. The antigen and phenol red were subjected to electrophoresis from the negative to the positive pole at high voltage (100 to 110 V, 5 V/cm) for 90 or 120 min. After the proteins were separated in the first dimension, the agarose gel was cut into 20 mm slabs. Each slab was transferred to a 70.7 mm by 70.7 mm glass plate. An intermediate gel of agarose was cast between the first dimension slab and a brass bar barrier (2). After the gel hardened, the barrier was removed and the intermediate gel was cut to 20 mm with a sharp blade. Agarose was mixed with antibody and was poured onto the remainder of the plate (30 mm). The antigen was subjected to electrophoresis from the negative to the positive pole at low voltage (50 to 60 V, 1.5 to 2 V/cm) for 18 h. After electrophoresis, the agarose gel was covered with filter paper and pressed under a heavy weight. The gel was washed in 0.1 M sodium chloride (NaCl) to remove nonprecipitated proteins, rinsed in deionized water to remove NaCl, and pressed. The gel was dried under a stream of warm air, stained in Coomassie brilliant blue (Bio-Rad Laboratories) in 45% ethyl alcohol - 10% glacial acetic acid, and destained in 45% ethyl alcohol - 10% glacial acetic acid to remove the background staining. After electrophoresis in the first dimension, the anodic edge of the phenol red marker of each well was measured and the value was set equal to 100%. The 100% value was used as a standard to calculate relative

migration velocity (RMV) of the other peaks. The immunoprecipitation peaks of the experimental gels were traced on tracing paper and measured from the anodic edge of the well to the center of each peak. The RMV was calculated and the peaks were numbered according to their relationship to the phenol red marker.

To visualize immunoprecipitation peaks from radioactively labeled antigens, the fluorographic procedure for thin layers was used (6). Two-methylnaphthalene [2-MN (Aldrich Chemical Company, Incorporated, Milwaukee, Wisconsin)] containing 0.4% 2,5-diphenyloxazole (Sigma Chemical Company) was melted at 34 to 36°C on a hot plate. The glass plate with the stained dried agarose was soaked in this mixture for approximately 30 to 45 sec until saturated. After the 2-MN had dried, the agarose was placed in contact with flashed X-Omat R X-ray film (Eastman Kodak Company, Rochester, New York) in a Kodak X-ray exposure holder between two pieces of masonite for pressing. The film was flashed following the procedure of Laskey and Mills (35), using a Kalt 280B electronic flash (Kalt Corporation, Santa Monica, California) with Kodak Wratten No. 22 gelatin filter and two pieces of Whatman No. 1 filter paper at a distance of 25 mm. The flashed side of the film was adjacent to the soaked agarose. The film was exposed for various intervals at -76°C and developed in an automatic processor.

Microneutralization Assay

In this procedure adapted from Rawls *et al.* (54), the immunoglobulin-rich fraction of each of the five antisera was tested against the homologous virus. The antisera were originally diluted 1:10 in maintenance medium followed by 11 twofold dilutions to a final dilution

of 1:2048. Virus dilutions made in maintenance medium contained 10^6 to 10^8 PFU/ml. From the virus dilution, 0.2 ml was added to 0.8 ml of maintenance medium for the virus control and to each antiserum dilution. For the antiserum control, 0.2 ml of the 1:10 dilution was added to 0.8 ml of maintenance medium. The mixtures were incubated in a 37°C water bath for 30 min. After incubation, 0.1 ml of each virus-antiserum mixture was added to RK-13 cells grown in 24 multiwell tissue culture plates (Falcon, Oxnard, California). After the antiserum-medium mixture, virus-medium mixture, and each virus-antiserum mixture were added to three wells, the plates were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 15 min. After virus adsorption, 1 ml of maintenance medium was added to each well and plates were incubated for 24 to 48 h or until one half of the virus control exhibited cytopathic effects. The cultures were examined and scored on the basis of 0 to 4+ cell destruction. The neutralization titer of each antiserum was calculated using the Kaerber Method (1).

RESULTS

To determine if there is a correlation between the antigenic composition of different strains of HSV and the ocular disease they cause, the antigenic composition of five strains of HSV-1 which cause characteristic and reproducible disease in the corneas of New Zealand white rabbits was determined. The kind of disease produced varied from epithelial (surface) only to a combination of epithelial and stromal (deep) disease (79). The severity of disease varied from mild to severe (8). The five HSV-1 strains used in this study caused epithelial disease with a variation in the severity: Shealy and RE, severe; McKrae and F, moderate; and CGA-3, mild. Four of the five strains also caused stromal disease and again there is variation in the severity: Shealy and RE, severe stromal necrosis with total corneal vascularization; McKrae and F, moderate stromal disease. CGA-3 does not cause stromal disease. The following experiments were done to see if a correlation between the antigenic composition of the five strains of HSV-1 and the ocular disease pattern exists.

Reference Patterns of Five Strains of Herpes Simplex Virus Type One

Cultures of HEp-2 cells were infected with each of the five strains that cause varying forms of eye disease and were radioactively labeled with ^{14}C-valine, -leucine, -isoleucine. The antigen preparation of each strain was subjected to electrophoresis against the homologous antiserum in cIEP and the immunoprecipitation peaks were

visualized by CBB staining and fluorography. Different volumes of antigen and antibody were used to determine the best resolution, and reproducible patterns were obtained with these different antigen/antibody ratios. As the ratio changed, the density and separation of the immunoprecipitation peaks also changed. The best resolution based on density and separation of immunoprecipitation peaks was established for the five strains using 20 to 45 ul antigen and 6 to 12 ul/cm^2 antibody. Immunoprecipitation peaks compared on the basis of RMV, shape and location were assigned the same antigen number.

To determine which of these antigens were virus-specific, cultures of HEp-2 cells were mock infected and radioactively labeled with ^{14}C-valine, -leucine, -isoleucine. The antigen preparation (15 ul) was subjected to electrophoresis against 10 ul/cm^2 of each of the five antisera, and a total of five peaks (two to five peaks with each antiserum) were resolved on the CBB stained gels and the fluorographs. These five peaks, designated A,B,C,D,E, were nonspecific. In the reference patterns of all five strains, all of the peaks resolved on the CBB stained gels were also resolved on the fluorographs except one or two peaks that matched peaks on the CBB stained gels of the HEp-2 mock infected cells with the same antiserum. Note A and B on Table 1 and Figure 2. In the reference patterns of all five strains, all of the peaks resolved on the fluorographs were virus-specific antigens except one to three peaks that matched peaks on the fluorographs of the HEp-2 mock infected cells with the same antiserum. Note C and D on Table 1 and Figure 2.

Twenty-three virus-specific antigens were resolved in the five strains and were classified as common, shared, and distinguishing. Three common antigens, designated c, were resolved in all five strains. Ten shared antigens, designated s, were resolved in more than one strain. Ten distinguishing antigens, designated d, were resolved in only one reference pattern. Antigens of the reference patterns of each of the five strains comparing the CBB stained gel and fluorography of each strain with that of the HEp-2 mock infected are shown in Figures 1-10. Tables 1-5 compare the RMV of these antigens and Table 6 summarizes these data.

The Shealy strain was designated as the prototype of HSV-1, since its reference pattern consists of the largest total number of antigens, 13, the largest number of shared antigens, ten, and no distinguishing antigens (Figures 1, 2; Table 1). Shealy, RE and McKrae strains showed very similar reference patterns of 13, 13, and 12 virus-specific antigens, respectively (Figures 1-6). RE and McKrae strains each shared ten antigens with Shealy and eight antigens with each other; RE strain had three distinguishing antigens and McKrae had two (Table 6). F strain, with 11 antigens, was less similar, sharing only eight antigens with Shealy strain, seven antigens with RE strain, and five antigens with McKrae strain, and having three distinguishing antigens (Figures 7, 8; Table 6). CGA-3 strain, with the fewest virus-specific antigens, eight, had two distinguishing antigens and the most distinct reference pattern. CGA-3 strain shared only six antigens with Shealy and McKrae strains, four antigens with RE strain, and three common antigens with F (Figures 9, 10; Table 6).

Cross-Reacting Antigens of Five Strains of Herpes Simplex Virus Type One

To further characterize the common, shared, and distinguishing antigens, experiments were done to determine cross-reacting antigens in which the ^{14}C-valine, -leucine, -isoleucine labeled antigen preparation of each strain was subjected to electrophoresis against the other four heterologous antisera. Each experiment was composed of one control and four experimental gels. The ratio of antigen and antiserum which was used to resolve the virus-specific antigens of each reference pattern was duplicated in the controls of these five experiments. In each experiment, the volume of antiserum used in the control gel was repeated in the other four experimental gels. The volume of antigen in the experimental gels was the same as that used to determine the reference patterns. The same nomenclature designating antigens of the reference patterns was chosen. Thirteen antigens which were not matched with one of the 23 virus-specific antigens were designated "U" for unknown. Only antigens resolved by fluorography were considered significant. Figures 11-20 show the cross-reacting antigens of these experiments. Tables 7-11 show the RMV of these antigens, and Table 12 summarizes these data.

These data show that the majority, 21 of the 23 virus-specific antigens, cross-react. Each antiserum resolved all but one or two of the virus-specific antigens of its homologous strain in at least one of the other four strains. Anti-Shealy did not resolve 5s or 6s (Table 7), anti-RE did not resolve 1d (Table 8), anti-McKrae did not resolve 9d (Table 9), and anti-F did not resolve 6s or 20d (Table 10) in any of

the four heterologous strains. Anti-CGA-3 resolved all of the virus-specific antigens of CGA-3 in at least two of the other four strains (Table 11). The three antigens resolved in each strain by all four antisera are the three common antigens resolved in all five reference patterns (2c, 21c, 22c) (Tables 7-11). The one exception, 2c, was not resolved by anti-F in the RE strain (Table 10). The ten shared antigens resolved in more than one reference pattern were resolved with more than one heterologous antiserum (Tables 7-11). Seven of these antigens (7s, 8s, 10s, 13s, 14s, 19s, 23s) were resolved in half or more than half of the 20 fluorographs, and three were found in less than half of the fluorographs (5s, 6s, 16s). Eight of these antigens were distinguishing antigens found in less than half of the 20 fluorographs (Tables 7-11). These distinguishing antigens (3d, 4d, 9d, 11d, 12d, 15d, 17d, and 18d) were found in the reference pattern of one strain only by the homologous antiserum or in that same strain with heterologous antisera or if present in a different strain, were resolved by the original homologous antiserum. Two distinguishing peaks, 1d of RE strain (Table 8) and 20d of F strain (Table 10), did not cross-react with any heterologous antisera.

Two points are important in the analysis of the cross-reactivity of one strain with one heterologous antiserum. First, what antigens that belong to the reference pattern of the control (homologous) antigen were resolved with the heterologous antiserum (antigens that cross-react), and second, what antigens that belong to the reference pattern of the heterologous antigen were resolved with the heterologous antiserum. Table 12 summarizes the data of the cross-reacting antigens

and supports the previous observation from analysis of the reference patterns showing the similarities and differences among these five strains. The four heterologous antisera resolved the largest number of cross-reacting antigens in the Shealy strain and the largest number of virus-specific antigens of the reference patterns in the Shealy strain. RE and McKrae strains were second and third respectively, showing many cross-reacting antigens. Strain F showed fewer cross-reacting antigens, and heterologous antisera with CGA-3 resolved the fewest cross-reacting antigens and the fewest antigens of the reference patterns in the CGA-3 strain. The data also showed the largest number of cross-reacting antigens between Shealy, RE and McKrae strains, several cross-reacting antigens between F strain and Shealy, RE, and McKrae strains and the fewest cross-reacting antigens between CGA-3 and the other four strains.

One interesting point is that each antiserum resolved antigens of a heterologous strain but did not resolve the same antigens in the reference pattern of the control strain. For example, 7s was resolved by anti-CGA-3 in all four heterologous strains but not in CGA-3. The corollary is also true, in that the other four antisera resolved 7s in the antigen preparation of the other four strains but not in the CGA-3 antigen preparation.

Glycosylated Antigen Patterns of Five Strains of Herpes Simplex Virus Type One

Since glycoproteins are important *in vivo* in disease and neutralization and *in vitro* in the kind of cytopathic effect (60,67,72,78), glycosylated virus-specific antigens were determined. Cultures of

HEp-2 cells were infected with each of the five strains and radioactively labeled with ^{14}C-glucosamine hydrochloride. Each strain was subjected to electrophoresis against the homologous antiserum, and reproducible patterns were obtained with different antigen/antibody ratios. Glycosylated antigen patterns were established using 60 to 120 ul antigen and 6 to 12 ul/cm^2 antibody. The same nomenclature designating antigens of the reference patterns and the cross-reacting patterns was used. Figures 21-30 illustrate these antigens, Tables 13-17 show the RMV of these antigens, and Table 18 summarizes these data.

To determine which of these antigens are virus-specific, cultures of HEp-2 cells were mock infected and radioactively labeled with ^{14}C-glucosamine. Sixty microliters of this antigen was subjected to electrophoresis against each of the five antisera, and a total of eight peaks were resolved on CBB staining (three to six with each antiserum). Five of these peaks were the same nonspecific A through E designated with the radioactive amino acid labeled HEp-2 mock infected cells plus three new peaks designated nonspecific, F, G and H. No peaks were resolved on the fluorographs of the HEp-2 mock infected cells. All the peaks resolved on CBB stained gels of the glycosylated antigen patterns were detected on fluorographs except two to five peaks that were nonspecific, i.e., that matched peaks on the CBB stained gel of the HEp-2 mock infected cells with the same antiserum. Note A,B,C,F,H of Table 13 and Figures 21 and 22. All of the peaks resolved by fluorography of each strain were virus specific except one or two peaks that matched peaks on the CBB stained gel of the HEp-2 mock

infected cells with the same antiserum. Note C of Table 14 and Figures 23 and 24.

Characteristic patterns were established with the homologous antiserum, and these patterns resemble the reference patterns of the ^{14}C-amino acid labeled strains. Strains F and CGA-3 were more like their own reference patterns than were Shealy, RE, and McKrae strains, in that seven glycosylated antigens of the 11 virus-specific antigens of F strain were resolved and five glycosylated antigens of the eight virus-specific antigens of CGA-3 were resolved. The majority, 14, of the virus-specific glycosylated antigens were identifiable with the 23 virus-specific antigens of the reference patterns (Table 18). The same point was illustrated by the ratio of the number of glycosylated virus-specific antigens matching the antigens of the reference pattern to the total number of glycosylated virus-specific antigens resolved in each strain: Shealy 8/9, RE 5/8, McKrae 8/11, F 7/7 and CGA-3 5/7. There were nine antigens designated "U" which were not identifiable on either the ^{14}C-amino acid labeled reference pattern of each strain or the ^{14}C-amino acid or -glucosamine labeled HEp-2 mock infected cells with each antiserum (Table 18). Two of the three common antigens, 21c and 22c, were glycosylated in all five strains, were resolved in all five reference patterns of ^{14}C-amino acid labeled strains, and were recognized by all four heterologous antisera in the cross-reacting experiments (Tables 6, 7-11, 18). Six of the ten shared antigens were glycosylated; 10s, 13s, 14s and 19s were resolved in at least three strains and 16s and 23s were resolved in two strains (Table 18). Six of the ten distinguishing antigens were glycosylated: 9d and 18d of

McKrae strain; 12d, 15d, and 20d of F strain and 3d of CGA-3 strain (Table 18).

Microneutralization Assay

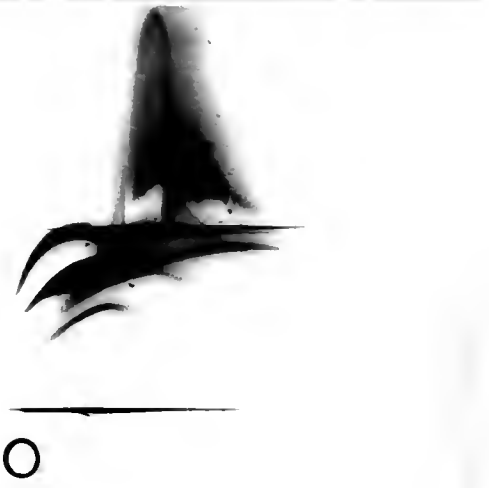
The neutralization titers of the five antisera used in cIEP were calculated using the Kaerber method. The log of the neutralization titer for each antiserum was anti-Shealy, -3.5; anti-McKrae, -3.5; anti-F, -5.5, anti-RE, -4.5; and anti-CGA-3, -0.833.

FIGURE 1

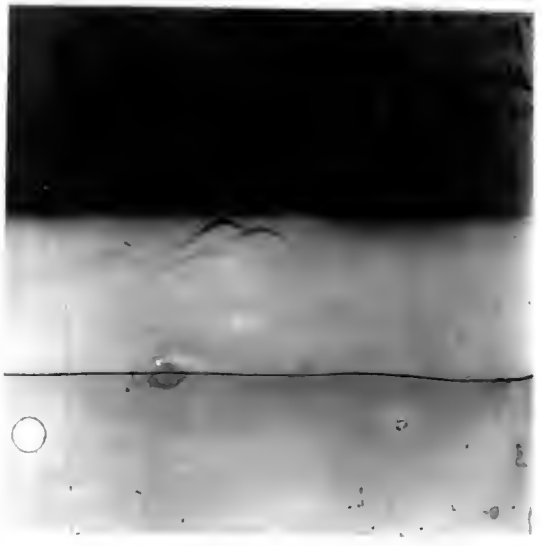
Crossed immunoelectrophoretic analysis of the reference pattern of the Shealy strain. Cultures of HEp-2 cells were infected with Shealy or mock infected and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin (Ig) rich fraction of antiserum to the Shealy strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 20 ul of Shealy antigen and 6 ul/cm^2 anti-Shealy Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-Shealy Ig. (D) Fluorography of C.

A

B



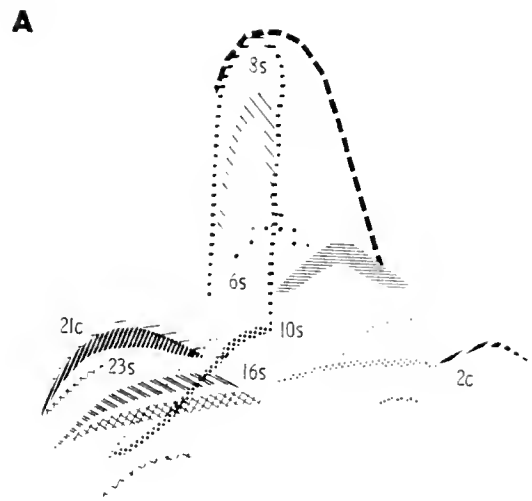
C

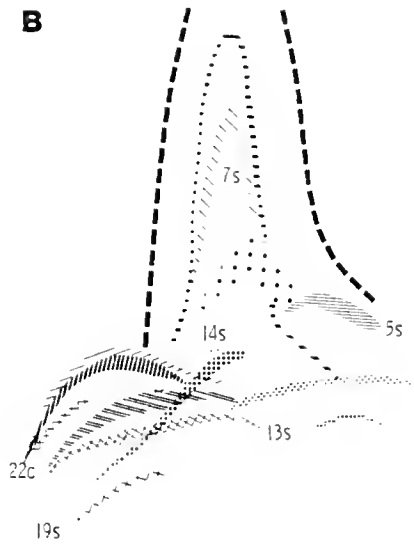


D

FIGURE 2

Tracing of Figure 1 of the reference pattern of the Shealy strain. (A) Coomassie brilliant blue staining of 20 ul of Shealy antigen and 6 ul/cm^2 anti-Shealy Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-Shealy Ig. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen.





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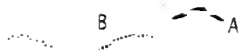

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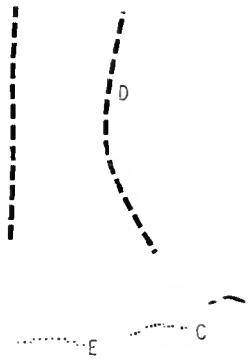


TABLE 1. REFERENCE PATTERN OF SHEALY

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-SHEALY¶ CBB§	RMV RANGE† FLUOROGRAPHY	HEp-2-MOCK INFECTED° CBB§	RMV FLUOROGRAPHY	VIRUS-SPECIFIC
A	67-69		69	70	-
B	54-56		57	57	-
2c*	45-55	54-79			+
C	55	50-56	55	54	-
5s	45-52	45-48			+
D	37	31-36		32	-
6s	33-36	33-35			+
7s	36	32-33			+
8s	32	31			+
10s	26-32	29-36			+
13s*	9-26	5-29			+
14s	22-27	25-27			+
16s*	12-24	13-24			+
E			22	27	-
19s	17-19	16-19			+
21c	12	10-14			+
22c	12	10-12			+
23s*	4-12	4-10			+
					13/18

•A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen

¶20 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with Shealy IEP antigen and 6 or 8 ul/cm^2 anti-Shealy Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-Shealy Ig

*Greater range due to broad peak

FIGURE 3

Crossed immunoelectrophoretic analysis of the reference pattern of the RE strain. Cultures of HEp-2 cells were infected with RE or mock infected and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin (Ig) rich fraction of antiserum to the RE strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 30 ul of RE antigen and 12 ul/cm^2 anti-RE Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-RE Ig. (D) Fluorography of C.

A

B

C

D

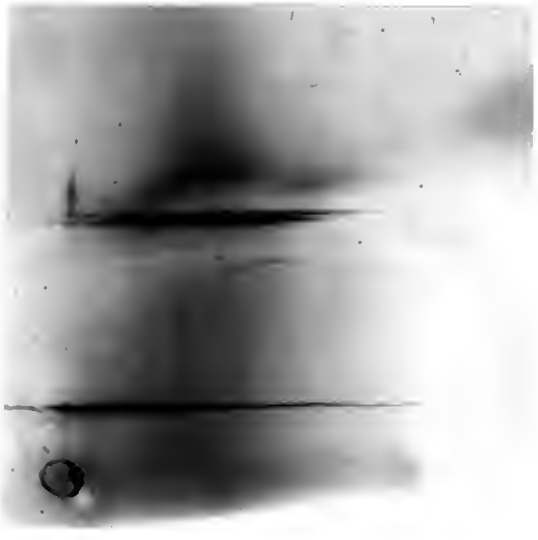
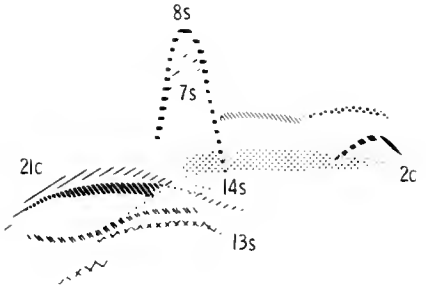


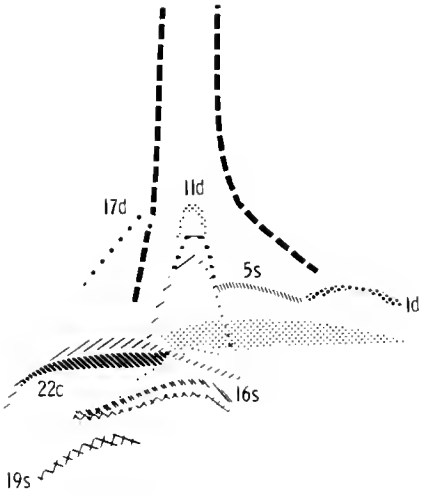
FIGURE 4

Tracing of Figure 3 of the reference pattern of the RE strain. (A) Coomassie brilliant blue staining of 30 ul of RE antigen and 12 ul/cm^2 anti-RE Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-RE Ig. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peaks 1d and 5s were not resolved in the gel in Figure 3A and 3B but were detected in at least two other gels of the reference pattern of the RE strain.

A



B



C

D

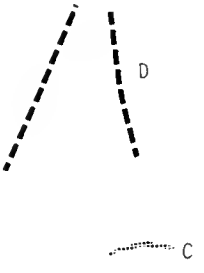


TABLE 2. REFERENCE PATTERN OF RE

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-RE¶ RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2-MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC
1d*	82	67-81			+
A	68-71		70		-
2c*	46-56	49-53			+
C			54	57	-
5s*	33	32-43			+
D		31-32		43	-
7s	31-33	28-32			+
8s	31-33	31-32			+
11d		31-32			+
13s*	22-31	18-31			+
14s	25-30	29-31			+
16s	26-29	28-30			+
E			27		-
17d		21-23			+
19s	15-20	13-22			+
21c	14-15	13-16			+
22c	13-16	14-19			+
					13/17

•A,C,D,E are nonspecific peaks; d is distinguishing antigen, c is common antigen, s is shared antigen

¶30 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with RE IEP antigen and 8 or 12 ul/cm^2 anti-RE Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-RE Ig

*Greater range due to broad peak

FIGURE 5

Crossed immunoelectrophoretic analysis of the reference pattern of the McKrae strain. Cultures of HEp-2 cells were infected with McKrae or mock infected and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin (Ig) rich fraction of antiserum to the McKrae strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 30 ul of McKrae antigen and 6 ul/cm^2 anti-McKrae Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-McKrae Ig. (D) Fluorography of C.

A

B

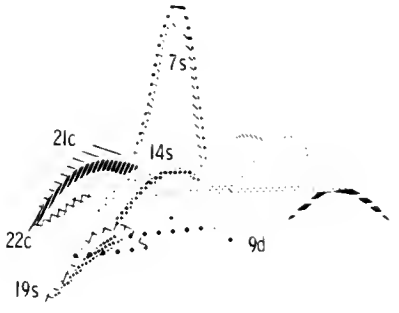
C

D

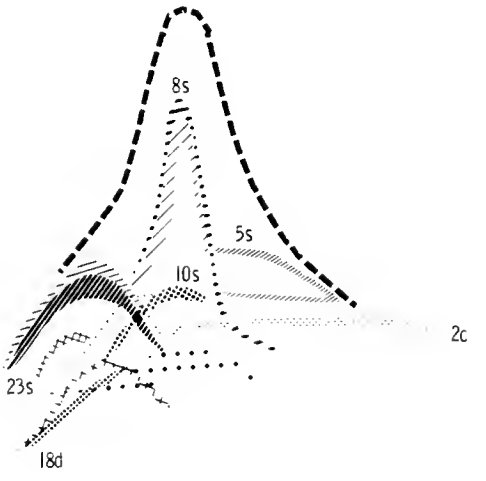
FIGURE 6

Tracing of Figure 5 of the reference pattern of the McKrae strain. (A) Coomassie brilliant blue staining of 30 ul of McKrae antigen and 6 ul/cm^2 anti-McKrae Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-McKrae Ig. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen.

A



B



C

D

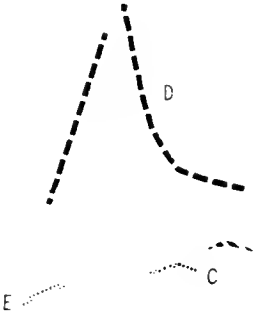


TABLE 3. REFERENCE PATTERN OF McKRAE

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-McKRAE¶ RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2-MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC
A	65-70		66	68	-
B	52-58		55	55	-
2c*	52-56	42-65			+
C			54	54	-
5s	42-44	40-46			+
D		28		32	-
7s	33-37	28-36			+
8s	30-37	27-35			+
9d	30-37	30-34			+
10s	30-37	29-33			+
14s	22-24	21-26			+
E			22	19	-
18d	16-18	19-21			+
19s	16-18	14-19			+
21c	14-16	12-15			+
22c	14-15	12-15			+
23s	13	11-16			+
					12/17

•A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen

¶15 or 30 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with McKrae IEP antigen and 6 ul/cm^2 anti-McKrae Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-McKrae Ig

*Greater range due to broad peak

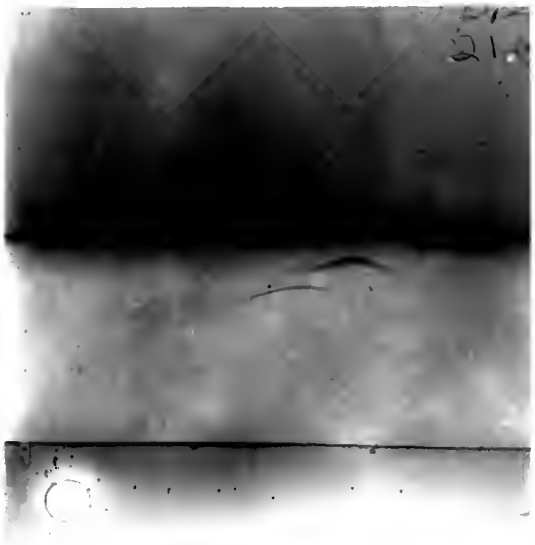
FIGURE 7

Crossed immunoelectrophoretic analysis of the reference pattern of the F strain. Cultures of HEp-2 cells were infected with F or mock infected and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 10 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the F strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 30 ul of F antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen. (D) Fluorography of C.

A

B

C



D

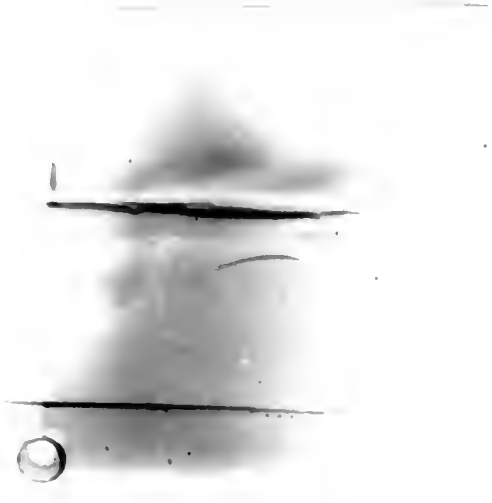
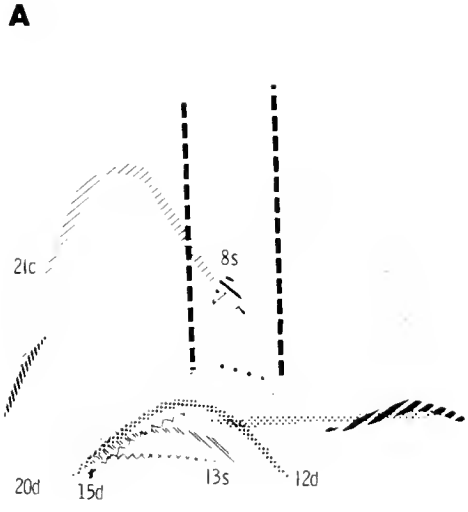
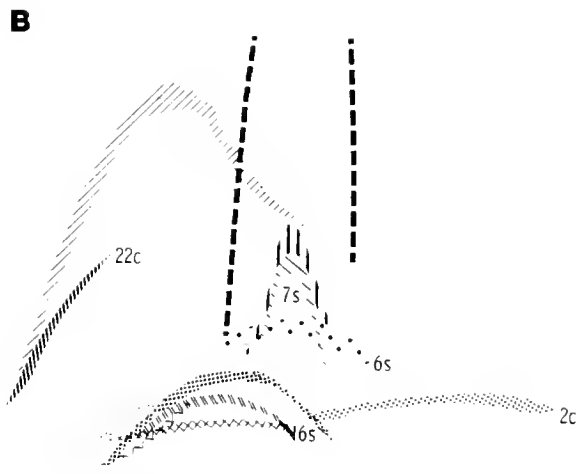
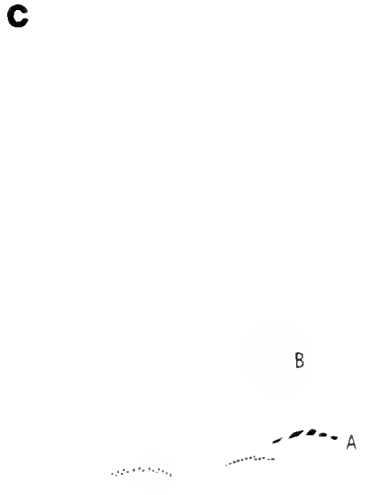


FIGURE 8

Tracing of Figure 7 of the reference pattern of the F strain. The reference gel contained 10 ul/cm^2 anti-F Ig. (A) Coomassie brilliant blue staining of 30 ul of F antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen.


A
21c
8s
20d
15d
13s
12d


B
22c
7s
6s
16s
2c


C
B
A

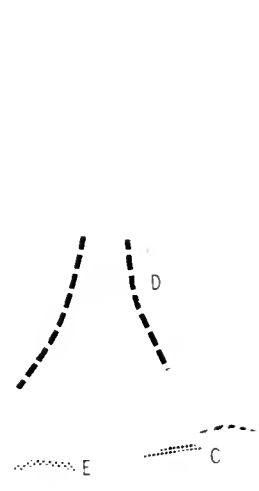

D
D
E
C

TABLE 4. REFERENCE PATTERN OF F

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-F¶ RMV CBB§	RANGE† FLUOROGRAPHY	HEp-2-MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC
A	66-67		68	72	-
B	56		56		-
2c*	44-54	48-59			+
C			54	56	-
D	33-37	33-37		42	-
6s	33-35	32-35			+
7s	33	32-33			+
8s	33-35	32-35			+
12d	26-27	25-32			+
13s*	19-33	19-33			+
15d	23-32	20-29			+
16s	17-23	22-25			+
E			21	19	-
20d	14	15			+
21c	7-16	9-16			+
22c*	(-3)-0	(-4)-11			+
					11/16

•A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen

¶30 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with F IEP antigen and 10 ul/cm^2 anti-F Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

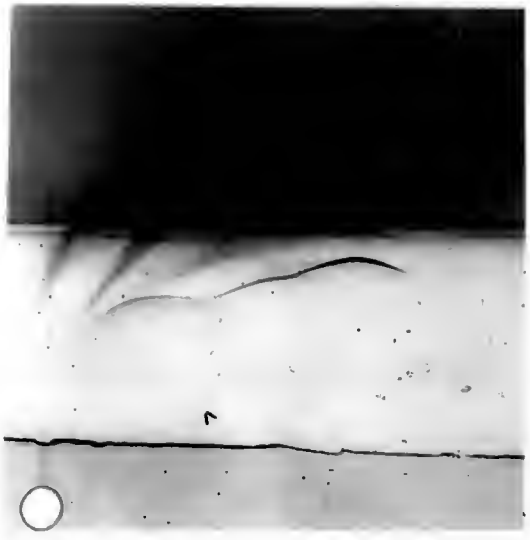
°15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-F Ig

*Greater range due to broad peak

FIGURE 9

Crossed immunoelectrophoretic analysis of the reference pattern of the CGA-3 strain. Cultures of HEp-2 cells were infected with CGA-3 or mock infected and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin (Ig) rich fraction of antiserum to the CGA-3 strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 45 ul of CGA-3 antigen and 10 ul/cm^2 anti-CGA-3 Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-CGA-3 Ig. (D) Fluorography of C.

A



B

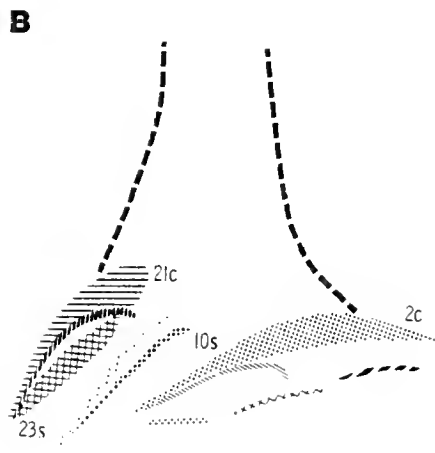
C

D

FIGURE 10

Tracing of Figure 9 of the reference pattern of the CGA-3 strain. (A) Coomassie brilliant blue staining of 45 ul of CGA-3 antigen and 10 ul/cm^2 anti-CGA-3 Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 15 ul of HEp-2 mock infected antigen and 10 ul/cm^2 anti-CGA-3 Ig. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen.


A
14s
22c
4d
3d


B
21c
2c
10s
23s


B
A

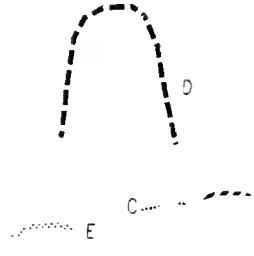

D
C
E

TABLE 5. REFERENCE PATTERN OF CGA-3

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-CGA-3¶ RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2-MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC
A	66-72	65-73	67	69	-
B	53-58		56		-
2c	52-60	55-61			+
C	59-62		54	57	-
3d	55-56	51-57			+
4d	41-46	37-46			+
D		31-39		40	-
10s	27-32	26-30			+
14s	27-33	24-31			+
E	24-25	22-30	21	23	-
21c	15-19	14-20			+
22c	11-15	12-15			+
23s	11	8-9			+
					8/13

•A,B,C,D,E are nonspecific peaks; c is common antigen, d is distinguishing antigen, s is shared antigen

¶45 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with CGA-3 IEP antigen and 10 ul/cm^2 anti-CGA-3 Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-CGA-3 Ig

TABLE 6. SUMMARY OF VIRUS-SPECIFIC ANTIGENS FROM FLUOROGRAPHY OF REFERENCE PATTERNS OF ^{14}C-VAL, -LEU, -ISL LABELED STRAINS

IMMUNOPPT. PEAK DESIGNATION	SHEALY	RE	McKRAE	F	CGA-3
1d*		X			
2c§	X	X	X	X	X
3d					X
4d					X
5s¶	X	X	X		
6s	X			X	
7s	X	X	X	X	
8s	X	X	X	X	
9d			X		
10s	X		X		X
11d		X			
12d				X	
13s	X	X		X	
14s	X	X	X		X
15d				X	
16s	X	X		X	
17d		X			
18d			X		
19s	X	X	X		
20d				X	
21c	X	X	X	X	X
22c	X	X	X	X	X
23s	X		X		X
Virus-specific Antigens	13	13	12	11	8

*d is distinguishing antigen
§c is common antigen
¶s is shared antigen

FIGURE 11

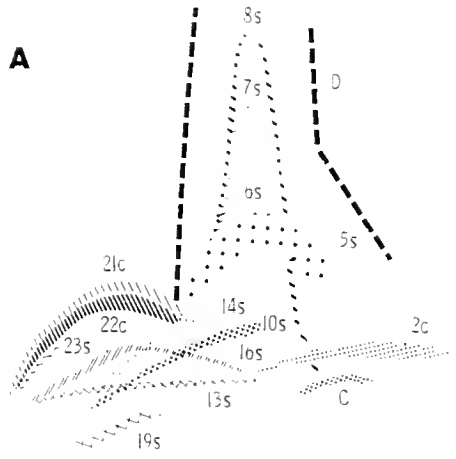
Crossed immunoelectrophoretic analysis of the pattern of cross-reacting antigens of the Shealy strain. Cultures of HEp-2 cells were infected with Shealy, RE, McKrae, F, or CGA-3 and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 6 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the Shealy strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. Fluorography of (A) 20 ul Shealy antigen, (B) 30 ul RE antigen, (C) 30 ul McKrae antigen, (D) 30 ul F antigen, (E) 45 ul CGA-3 antigen.

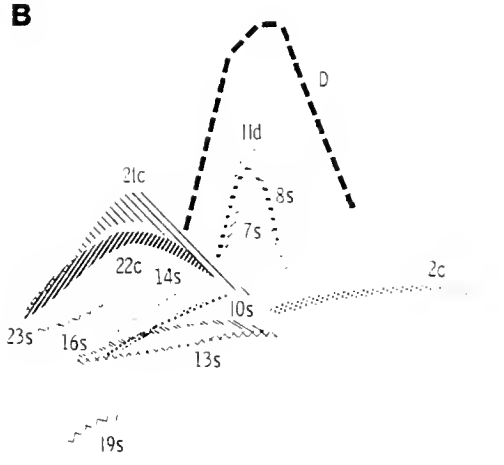
C

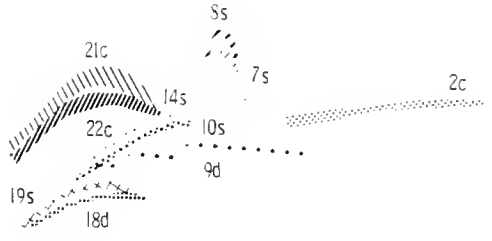
E

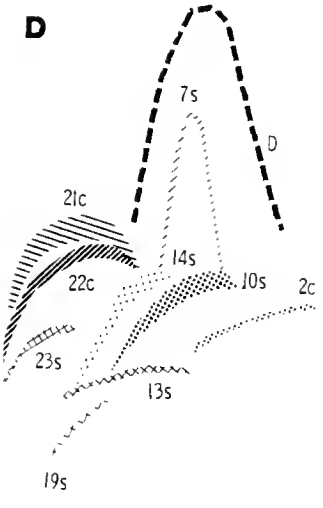
FIGURE 12

Tracing of Figure 11 of the pattern of cross-reacting antigens of the Shealy strain. Fluorography of 6 ul/cm^2 anti-Shealy Ig and (A) 20 ul Shealy antigen, (B) 30 ul RE antigen, (C) 30 ul McKrae antigen, (D) 30 ul F antigen, (E) 45 ul CGA-3 antigen. Immunoprecipitation peaks labeled: C and D are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peak C was not resolved in the gel in Figure 11A but was detected in at least two other gels of the reference pattern of the Shealy strain.


A
8s
7s
D
6s
5s
21c
14s
22c
10s
2c
23s
16s
13s
C
19s


B
D
11d
21c
8s
7s
22c
14s
2c
10s
23s
16s
13s
19s


C
8s
21c
7s
2c
14s
22c
10s
9d
19s
18d


D
7s
D
21c
14s
22c
10s
2c
23s
13s
19s

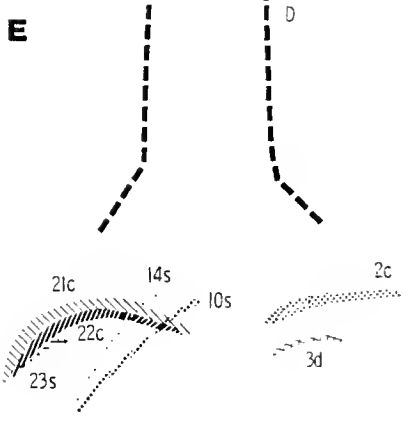

E
D
21c
14s
10s
2c
22c
23s
3d

TABLE 7. CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM: ANTI-SHEALY Ig

	IMMUNOPPT. PEAK DESIGNATION*	SHEALY†	RE†	McKRAE†	F†	CGA-3†
	2c*	40-59°	43-60°	43-60°	44-56°	52-63°
ANTIGENS THAT	C	( )°				
CROSS-REACT	5s	45°				
WITH	D	31°	35°		36°	26°
ANTIGENS OF	6s	35°				
THE REFERENCE	7s	32°	33°	32°	33°	
PATTERN OF	8s	31°	33°	32°		
THE HOMOLOGOUS	10s	30°	29	22°	33	23°.
VIRUS	13s*	5-29°	24-29°		18-29°	
[SHEALY]	14s	25°	24°	22°	24	21°
	16s*	10-19°	19-29°			
	19s	16°	9°	14°	20	
	21c	14°	16°	16°	13°	13°
	22c	12°	16°	16°	14°	13°
	23s	4°	8		10	8°
ANTIGENS OF	3d					52°
THE REFERENCE	9d			34°		
PATTERN OF THE	11d		33°			
HETEROLOGOUS	18d			17°		
VIRUS						
TOTAL NUMBER OF PEAKS:		14	13	10	10	8
VIRUS-SPECIFIC ANTIGENS THAT CROSS-REACT WITH SHEALY:			11	8	9	6
VIRUS-SPECIFIC ANTIGENS OF EACH REFERENCE PATTERN:		13/13	11/13	10/12	5/11	7/8

•C and D are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen

†Relative migration velocity of the immunoprecipitation peaks of ^{14}C-val, -leu, -isl IEP antigen against 6 ul/cm^2 anti-Shealy Ig resolved on fluorography: 20 ul Shealy; 30 ul RE, McKrae, F; 45 ul CGA-3 antigen.

°Antigens of the reference pattern of each virus

()Antigen of the Shealy reference pattern not detected in this gel

*Greater range due to broad peak

FIGURE 13

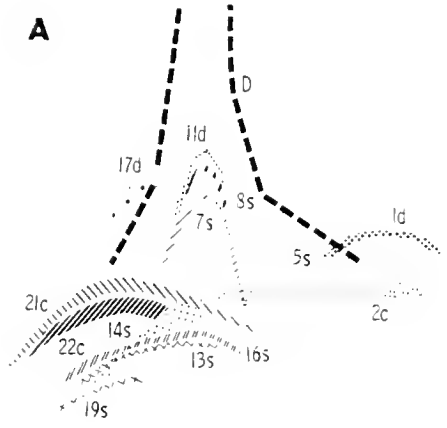
Crossed immunoelectrophoretic analysis of the pattern of cross-reacting antigens of the RE strain. Cultures of HEp-2 cells were infected with RE, Shealy, McKrae, F, or CGA-3 and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 12 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the RE strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. Fluorography of (A) 30 ul RE antigen, (B) 20 ul Shealy antigen, (C) 30 ul McKrae antigen, (D) 30 ul F antigen, (E) 45 ul CGA-3 antigen.

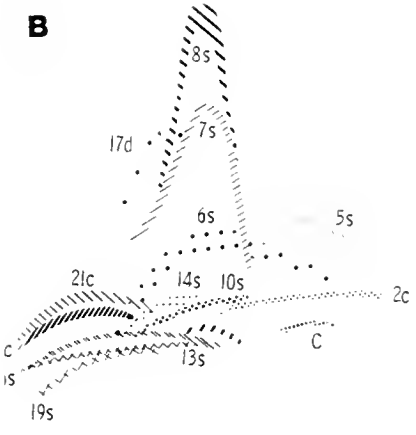
C

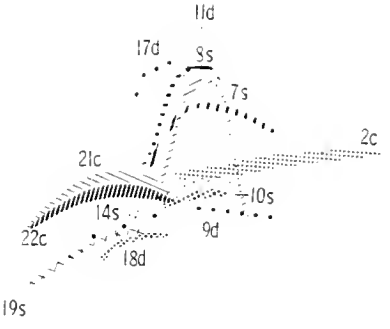
E

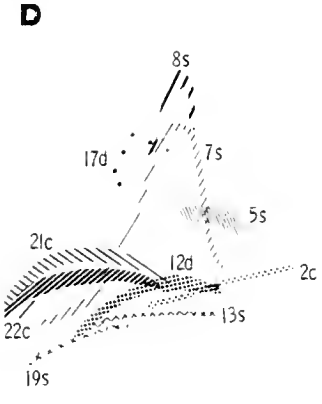
FIGURE 14

Tracing of Figure 13 of the pattern of cross-reacting antigens of the RE strain. Fluorography of 12 ul/cm^2 anti-RE Ig and (A) 30 ul RE antigen, (B) 20 ul Shealy antigen, (C) 30 ul McKrae antigen, (D) 30 ul F antigen, (E) 45 ul CGA-3 antigen. Immunoprecipitation peaks labeled: C and D are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peaks which are not labeled are unknown antigens.


A
D
11d
17d
8s
7s
1d
5s
21c
2c
14s
22c
13s
16s
19s


B
8s
17d
7s
6s
5s
21c
14s
10s
2c
C
13s
19s


C
11d
17d
8s
7s
2c
21c
10s
14s
9d
22c
18d
19s


D
8s
17d
7s
5s
21c
12d
2c
13s
22c
19s

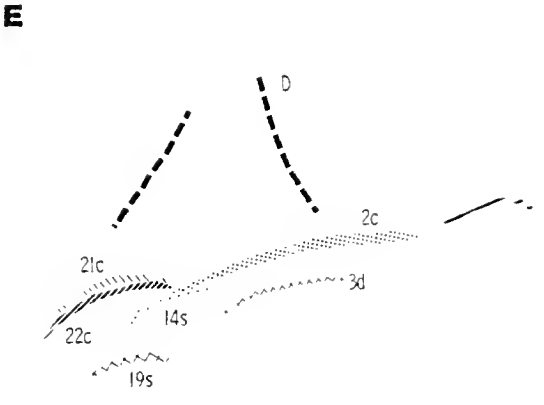

E
D
2c
21c
3d
14s
22c
19s

TABLE 8. CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM: ANTI-RE Ig

	IMMUNOPPT. PEAK DESIGNATION•	RE†	SHEALY†	McKRAE†	F†	CGA-3†
	1d	67-71°				
	2c*	44-58°	47-62°	44-62°	58°	58-70°
ANTIGENS THAT	5s	43°	51°		35	
CROSS-REACT	D	32°				37°
WITH	7s	32°	39°	31°	31°	
ANTIGENS OF	8s	31°	33°	29°	31°	
THE REFERENCE	11d	31°		31		
PATTERN OF	13s*	18-31°	11-29°		23-33°	
THE HOMOLOGOUS	14s	29°	31°	31°		30°
VIRUS	16s*	29°	11-22°			
[RE]	17d	23°	22	22	19	
	19s	18°	20°	16°	19	19°
	21c	13°	11°	14°	14°	19°
	22c	17°	11°	16°	19°	19°
ANTIGENS OF	C		58°			
THE REFERENCE	3d					53°
PATTERN OF THE	6s		37°			
HETEROLOGOUS	9d			30°		
VIRUS	10s		31°	31°		
	12d				26°	
	18d			18°		
UNKNOWN	U		31			
ANTIGENS	U			31		
	U					93
TOTAL NUMBER OF PEAKS:		14	15	13	10	8
VIRUS-SPECIFIC ANTIGENS THAT CROSS-REACT WITH RE:			11	9	9	5
VIRUS-SPECIFIC ANTIGENS OF EACH REFERENCE PATTERN:		13/13	12/13	10/12	7/11	6/8

•D and C are nonspecific peaks; d is distinguishing antigen, c is common antigen, s is shared antigen, U is unknown antigen

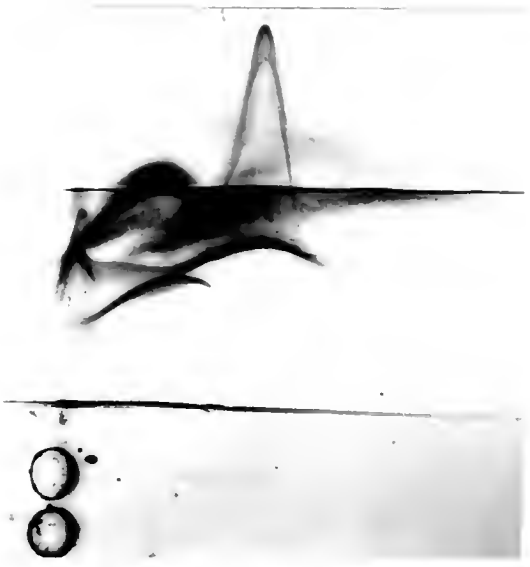
†Relative migration velocity of the immunoprecipitation peaks of ^{14}C-val, -leu, -isl IEP antigen against 12 ul/cm^2 anti-RE Ig resolved on fluorography: 20 ul Shealy; 30 ul RE, McKrae, F; 45 ul CGA-3 antigen.

°Antigens of the reference pattern of each virus

*Greater range due to broad peak

FIGURE 15

Crossed immunoelectrophoretic analysis of the pattern of cross-reacting antigens of the McKrae strain. Cultures of HEp-2 cells were infected with McKrae, Shealy, RE, F, or CGA-3 and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 6 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the McKrae strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. Fluorography of (A) 30 ul McKrae antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul F antigen, (E) 60 ul CGA-3 antigen.

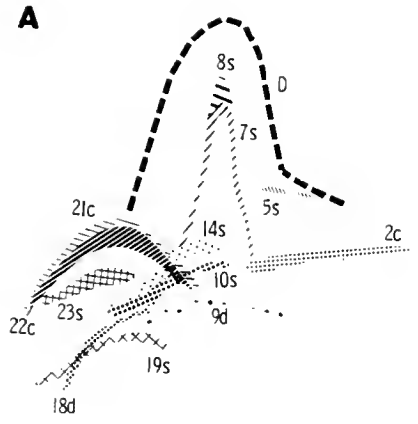


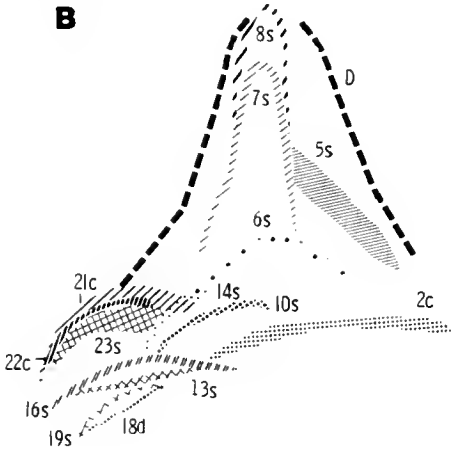
C

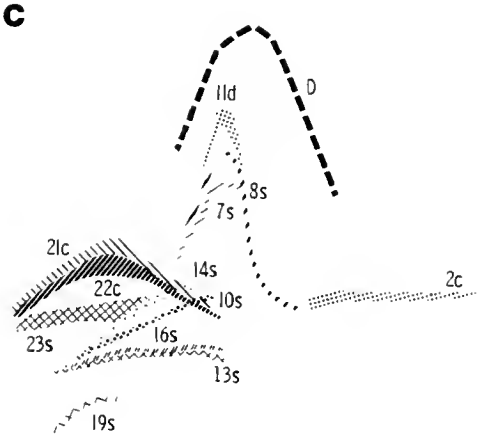
E

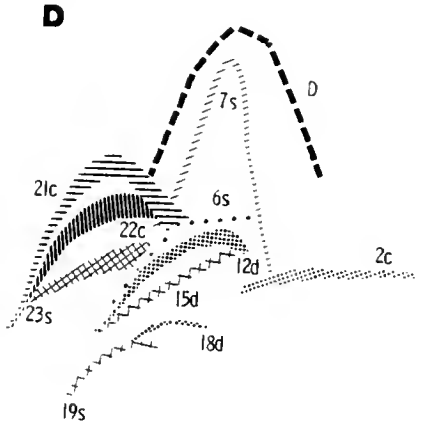
FIGURE 16

Tracing of Figure 15 of the pattern of cross-reacting antigens of the McKrae strain. Fluorography of 6 ul/cm^2 anti-McKrae Ig and (A) 30 ul McKrae antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul F antigen, (E) 60 ul CGA-3 antigen. Immunoprecipitation peaks labeled: D is a nonspecific peak; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peak 18d was not resolved in the gel in Figure 15A but was detected in at least two other gels of the reference pattern of the McKrae strain. Immunoprecipitation peaks which are not labeled are unknown antigens.


A
8s
D
7s
5s
21c
14s
2c
10s
23s
22c
9d
19s
18d


B
8s
D
7s
5s
6s
21c
14s
10s
2c
23s
22c
13s
16s
19s
18d


C
11d
D
8s
7s
21c
14s
22c
10s
2c
23s
16s
13s
19s


D
7s
D
21c
6s
22c
12d
2c
15d
23s
18d
19s

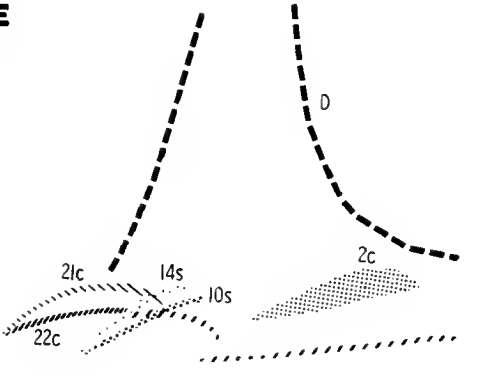

E
D
21c
14s
2c
10s
22c

TABLE 9. CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM: ANTI-McKRAE Ig

	IMMUNOPPT. PEAK DESIGNATION•	McKRAE†	SHEALY†	RE†	F†	CGA-3†
	2c*	44-65°	37-56°	40-60°	64°	63°
ANTIGENS THAT	5s	46°	37°			
CROSS-REACT	D	( )°	33°	36°	38°	39°
WITH	7s	35°	32°	34°	36°	
ANTIGENS OF	8s	35°	32°	34°		
THE REFERENCE	9d	34°				
PATTERN OF	10s	35°	25°	30		31°
THE HOMOLOGOUS	14s	26°	20°	28°		29°
VIRUS	18d	( )°	15		29	
[McKRAE]	19s	19°	15°	14°	19	
	21c	15°	11°	16°	14°	20°
	22c	15°	9°	16°	16°	20°
	23s	15°	9°	16	16	
ANTIGENS OF	6s		32°		33°	
THE REFERENCE	11d			32°		
PATTERN OF THE	12d				33°	
HETEROLOGOUS	13s		15-22°	26-34°		
VIRUS	15d				33°	
	16s		11-17°	26-34°		
UNKNOWN	U*					61-82
ANTIGENS	U					31
TOTAL NUMBER OF PEAKS:		11	15	13	11	8
VIRUS-SPECIFIC ANTIGENS THAT CROSS-REACT WITH McKRAE:			11	9	7	5
VIRUS-SPECIFIC ANTIGENS OF EACH REFERENCE PATTERN:		11/12	13/13	10/13	7/11	5/8

•D is a nonspecific peak; c is common antigen, s is shared antigen, d is distinguishing antigen, U is unknown antigen

†Relative migration velocity of the immunoprecipitation peaks of ^{14}C-val, -leu, -isl IEP antigen against 6 ul/cm^2 anti-McKrae Ig resolved on fluorography: 20 ul Shealy; 30 ul McKrae, RE, F; 60 ul CGA-3 antigen.

°Antigens of the reference pattern of each virus

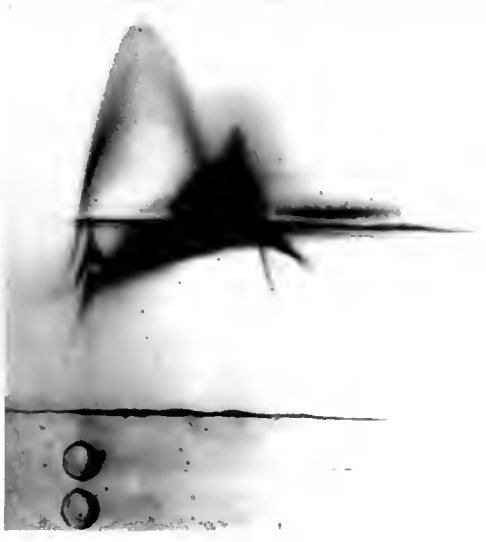
()Antigens of the McKrae reference pattern not detected in this gel

*Greater range due to broad peak

FIGURE 17

Crossed immunoelectrophoretic analysis of the pattern of cross-reacting antigens of the F strain. Cultures of HEp-2 cells were infected with F, Shealy, RE, McKrae, or CGA-3 and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 10 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the F strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. Fluorography of (A) 30 ul F antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul McKrae antigen, (E) 60 ul CGA-3 antigen.

C



E

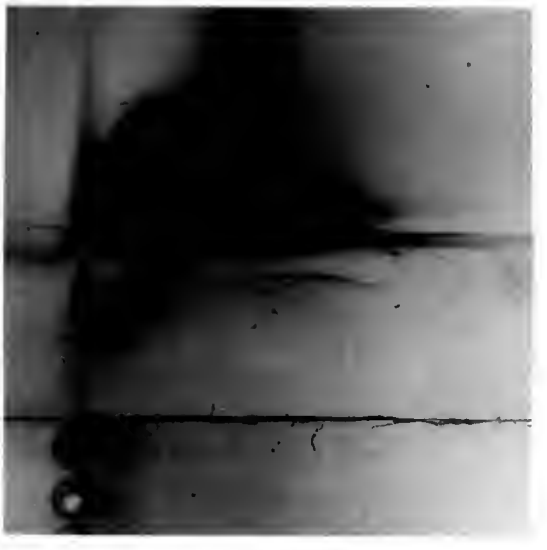
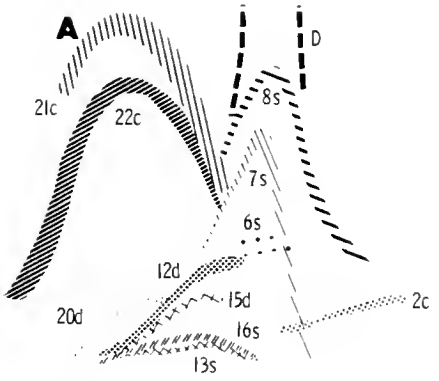
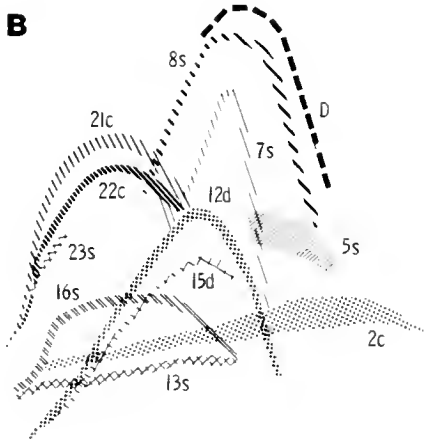
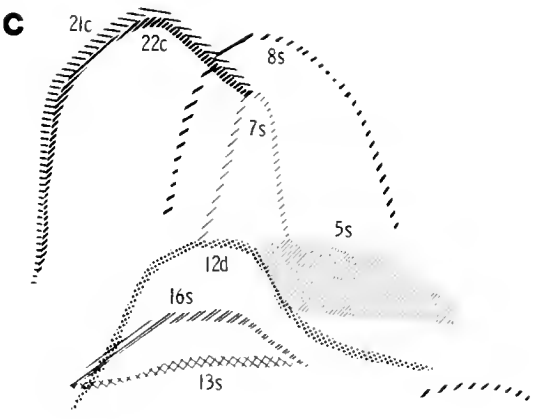


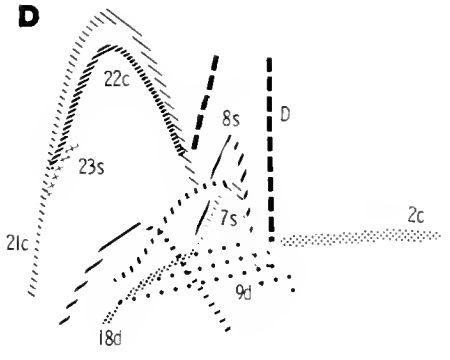
FIGURE 18

Tracing of Figure 17 of the pattern of cross-reacting antigens of the F strain. Fluorography of 10 ul/cm^2 anti-F Ig and (A) 30 ul F antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul McKrae antigen, (E) 60 ul CGA-3 antigen. Immunoprecipitation peaks labeled: A and D are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peak 20d was not resolved in the gel in Figure 17A but was detected in at least two other gels of the reference pattern of the F strain. Immunoprecipitation peaks which are not labeled are unknown antigens.


A
D
8s
21c
22c
7s
6s
12d
15d
20d
16s
2c
13s


B
8s
D
21c
7s
22c
12d
5s
23s
15d
16s
2c
13s


C
21c
22c
8s
7s
5s
12d
16s
13s


D
22c
8s
D
23s
7s
2c
21c
9d
18d

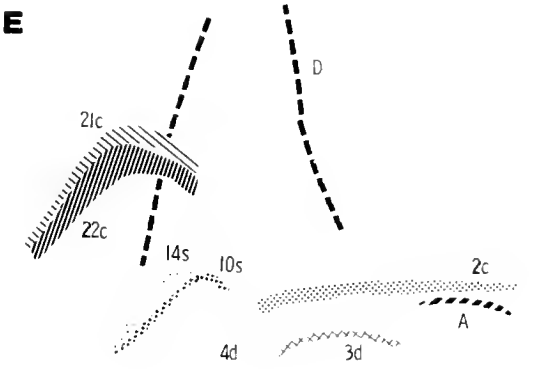

E
D
21c
22c
14s
10s
2c
A
4d
3d

TABLE 10. CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM: ANTI-F Ig

	IMMUNOPPT. PEAK DESIGNATION*	F†	SHEALY†	RE†	McKRAE†	CGA-3†
ANTIGENS THAT CROSS-REACT WITH ANTIGENS OF THE REFERENCE PATTERN OF THE HOMOLOGOUS VIRUS [F]	2c*	59°	33-56°		40-62°	42-70°
	D	35°	31°		31°	35°
	6s	35°				
	7s	32°	31°	28°	31°	
	8s	35°	31°	26°	31°	
	12d	25°	28	20		
	13s	22°	24°	22°		
	15d	22°	26			
	16s	22°	24°	22°		
	20d	( )°				
	21c	9°	10°	12°	9°	16°
	22c	11°	11°	12°	9°	19°
ANTIGENS OF THE REFERENCE PATTERN OF THE HETEROLOGOUS VIRUS	A					70°
	3d					58°
	4d					49°
	5s		38°	43°		
	9d				31°	
	10s					26°
	14s					23°
	18d				22°	
	23s		1°		3°	
UNKNOWN ANTIGENS	U			70		
	U				27	
	U				11	
TOTAL NUMBER OF PEAKS:		11	12	9	11	9
VIRUS-SPECIFIC ANTIGENS THAT CROSS-REACT WITH F:			9	7	5	3
VIRUS-SPECIFIC ANTIGENS OF EACH REFERENCE PATTERN:		10/11	9/13	7/13	8/12	7/8

*D and A are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen, U is unknown antigen

†Relative migration velocity of the immunoprecipitation peaks of ^{14}C-val, -leu, -isl IEP antigen against 10 ul/cm^2 anti-F Ig resolved on fluorography: 20 ul Shealy; 30 ul F, RE, McKrae; 60 ul CGA-3 antigen.

°Antigens of the reference pattern of each virus

()Antigen of the F reference pattern not detected in this gel

*Greater range due to broad peak

FIGURE 19

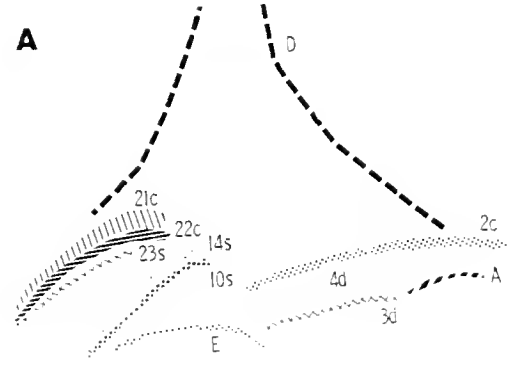
Crossed immunoelectrophoretic analysis of the pattern of cross-reacting antigens of the CGA-3 strain. Cultures of HEp-2 cells were infected with CGA-3, Shealy, RE, McKrae, or F and grown in medium supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 10 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the CGA-3 strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. Fluorography of (A) 45 ul CGA-3 antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul McKrae antigen, (E) 30 ul F antigen.

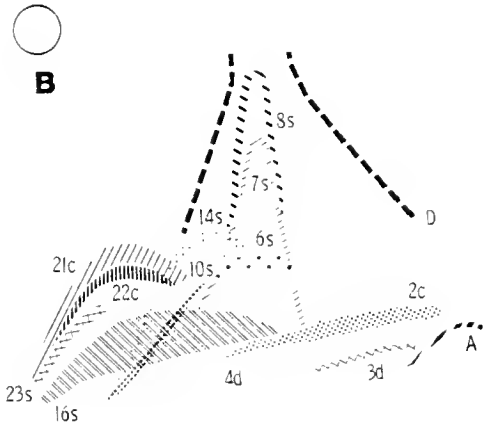
C

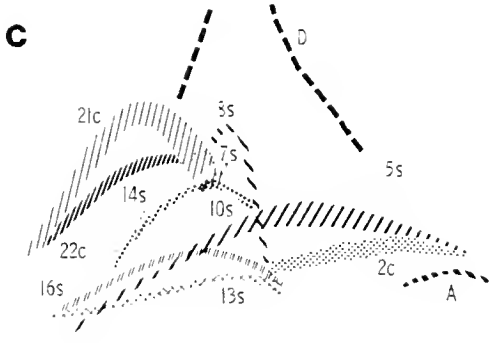
E

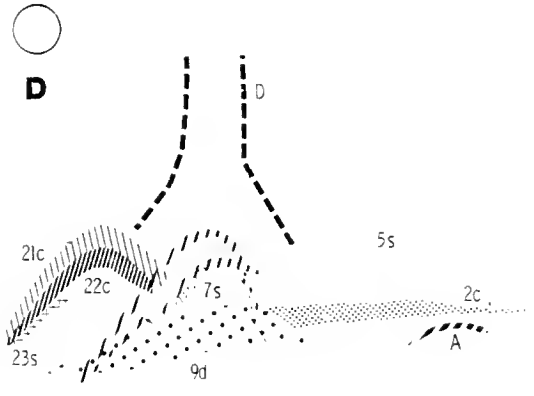
FIGURE 20

Tracing of Figure 19 of the pattern of cross-reacting antigens of the CGA-3 strain. Fluorography of 10 ul/cm^2 anti-CGA-3 Ig and (A) 45 ul CGA-3 antigen, (B) 20 ul Shealy antigen, (C) 30 ul RE antigen, (D) 30 ul McKrae antigen, (E) 30 ul F antigen. Immunoprecipitation peaks labeled: A,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peaks which are not labeled are unknown antigens.


A
D
21c
22c
14s
23s
10s
2c
4d
A
3d
E


B
8s
7s
14s
6s
D
21c
10s
22c
2c
A
4d
3d
23s
16s


C
D
21c
5s
7s
5s
14s
10s
22c
2c
16s
13s
A


D
D
21c
5s
22c
7s
2c
A
23s
9d

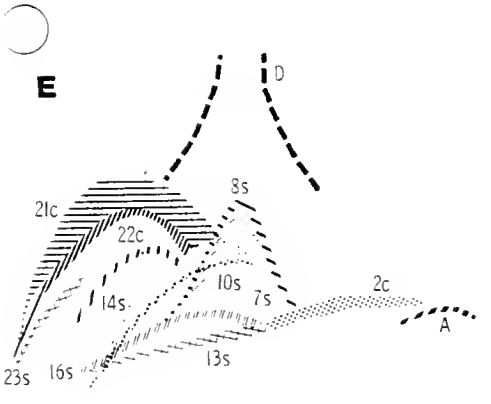

E
D
8s
21c
22c
10s
7s
2c
14s
A
13s
23s
16s

TABLE 11. CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM: ANTI-CGA-3 Ig

	IMMUNOPPT. PEAK DESIGNATION•	CGA-3†	SHEALY†	RE†	McKRAE†	F†
	A	65°	67	69	67	65
ANTIGENS THAT	2c*	51-61°	53-59°	43-64°	52-63°	50-56°
CROSS-REACT	3d	51°	54			
WITH	4d	37°	46			
ANTIGENS OF	D	29°	37°	40°	33°	33°
THE REFERENCE	10s	27°	18°	28		28
PATTERN OF	14s	24°	25°	26°		26
THE HOMOLOGOUS	E	22°				
VIRUS	21c	14°	10°	18°	13°	13°
[CGA-3]	22c	14°	10°	18°	13°	14°
	23s	10°	5°		6°	10
	5s			45°	44°	
ANTIGENS OF	6s		33°			
THE REFERENCE	7s		33°	34°	33°	31°
PATTERN OF THE	8s		33°	34°		31°
HETEROLOGOUS	9d				33°	
VIRUS	13s			30°		25°
	16s		18°	30°		25°
	U			47		
UNKNOWN	U				33	
ANTIGENS	U				29	
	U					15
	U				13	
TOTAL NUMBER OF PEAKS:		11	14	13	12	13
VIRUS-SPECIFIC ANTIGENS THAT CROSS-REACT WITH CGA-3:			8	5	4	6
VIRUS-SPECIFIC ANTIGENS OF EACH REFERENCE PATTERN:		8/8	10/13	9/13	7/12	7/11

•A,D and E are nonspecific peaks; c is common antigen, d is distinguishing antigen, s is shared antigen, U is unknown antigen

†Relative migration velocity of the immunoprecipitation peaks of ^{14}C-val, -leu, -isl IEP antigen against 10 ul/cm^2 anti-CGA-3 Ig resolved on fluorography: 20 ul Shealy; 30 ul RE, McKrae, F; 45 ul CGA-3 antigen.

°Antigens of the reference pattern of each virus

*Greater range due to broad peak

TABLE 12. SUMMARY OF VIRUS-SPECIFIC ANTIGENS FROM FLUOROGRAPHY OF CROSS-REACTING ANTIGENS WITH HETEROLOGOUS ANTISERUM

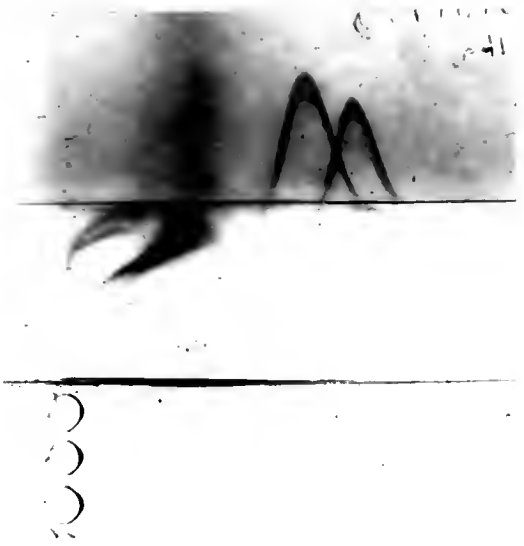
	SHEALY†	RE†	McKRAE†	F†	CGA-3†
Virus-specific antigens of each reference pattern with homologous antiserum	13	13	12	11	8
Virus-specific antigens that cross-react*					
anti-SHEALY		11	8	9	6
anti-RE	11		9	9	5
anti-McKRAE	11	9		7	5
anti-F	9	7	5		3
anti-CGA-3	8	5	4	6	
Virus-specific antigens of each reference pattern with heterologous antiserum					
anti-SHEALY		11	10	5	7
anti-RE	12		10	7	6
anti-McKRAE	13	10		7	5
anti-F	9	7	8		7
anti-CGA-3	10	9	7	7	

†^{14}C-val, -leu, -isl labeled antigen subjected to electrophoresis against four heterologous antisera. Immunoprecipitation peaks were resolved by fluorography.
*Antigens that belong to the reference pattern of the control antigen that were resolved with heterologous antiserum.

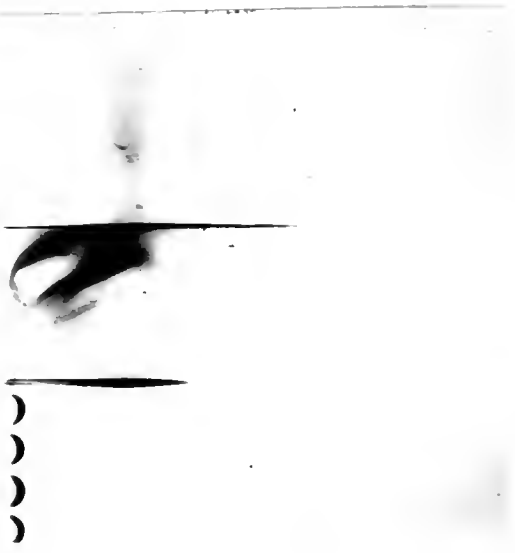
FIGURE 21

Crossed immunoelectrophoretic analysis of the pattern of glycosylated antigens of the Shealy strain. Cultures of HEp-2 cells were infected with Shealy or mock infected and grown in medium supplemented with ^{14}C-glucosamine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 6 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the Shealy strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 120 ul of Shealy antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C.

A



B



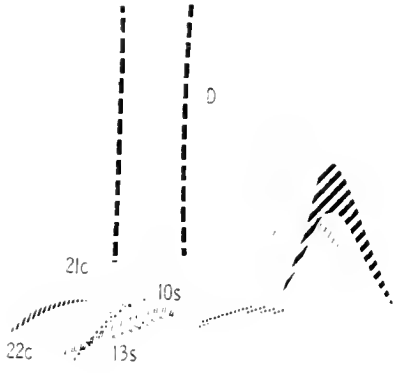
C

D

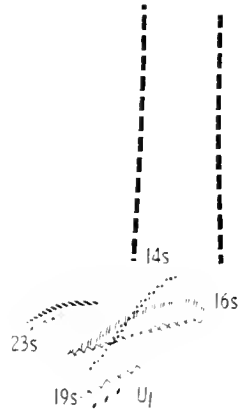
FIGURE 22

Tracing of Figure 21 of the pattern of glycosylated antigens of the Shealy strain. The reference gel contained 6 ul/cm^2 anti-Shealy Ig. (A) Coomassie brilliant blue staining of 120 ul of Shealy antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,F,H are nonspecific peaks; c is common antigen, s is shared antigen; U is unknown antigen. Immunoprecipitation peak F was not resolved in the gel in Figure 21A but was detected in at least two other gels of the reference pattern of the glycosylated antigen pattern of the Shealy strain.

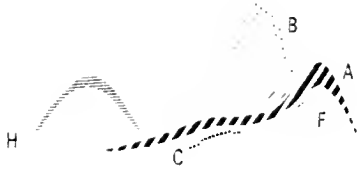
A



B



C



D

TABLE 13. GLYCOSYLATED ANTIGEN PATTERN OF SHEALY

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-SHEALY RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2 MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS
A	64-70		67-69*		-
F	63		61		-
B	52-59		57*		-
C	48-52		52		-
D	26	28			-
10s	25-33	27-33			+
13s	25-32	28-30			+
14s	25-27	27-30			+
16s	25-32	26-30			+
H	21-29		22*		-
19s		17-22			+
21c	9-17	9-18			+
22c	9-17	9-18			+
23s		7			+
U_1		17-20			+
					9/15

•A,B,C,D,F,H are nonspecific peaks; s is shared antigen, c is common antigen, U is unknown antigen

¶30, 90, 120 ul ^{14}C-glucosamine labeled HEp-2 infected with Shealy IEP antigen and 6 ul/cm^2 anti-Shealy Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°60 ul ^{14}C-glucosamine labeled HEp-2 mock infected IEP antigen and 6 ul/cm^2 anti-Shealy Ig

*RMV range of complex of 2 or 3 peaks

FIGURE 23

Crossed immunoelectrophoretic analysis of the pattern of glycosylated antigens of the RE strain. Cultures of HEp-2 cells were infected with RE or mock infected and grown in medium supplemented with ^{14}C-glucosamine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 12 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the RE strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 120 ul of RE antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C.

A

B

C

D

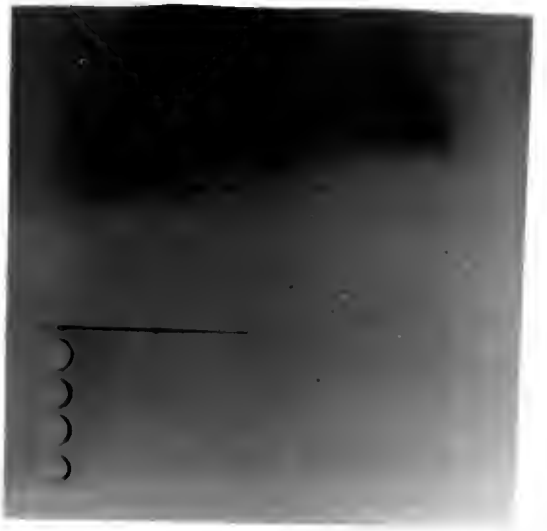
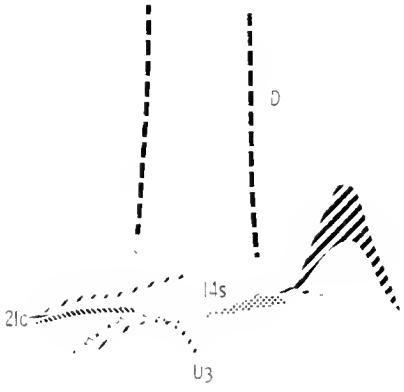


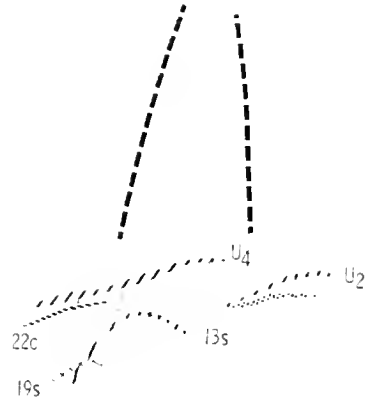
FIGURE 24

Tracing of Figure 23 of the pattern of glycosylated antigens of the RE strain. Reference gel contained 12 ul/cm^2 anti-RE Ig. (A) Coomassie brilliant blue staining of 120 ul of RE antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,C,D,H are nonspecific peaks; c is common antigen, s is shared antigen; U is unknown antigen.

A



B



C

D

TABLE 14. GLYCOSYLATED ANTIGEN PATTERN OF RE

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-RE RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2 MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS
A	67-71		67-68*		-
C	52-57	51-58	52		-
D	37	36-45			-
13s	26-31	24-30			+
14s	32-35	33-38			+
H	20-26		22-25*		-
19s		15			+
21c	17-20	16-20			+
22c	17-20	18-20			+
U_2	55-57	57-58			+
U_3	25-26	24-25			+
U_4	22	22			+
					8/12

•A,C,D,H are nonspecific peaks; s is shared antigen, c is common antigen, U is unknown antigen

¶60 or 120 ul ^{14}C-glucosamine labeled HEp-2 infected with RE IEP antigen and 12 or 15 ul/cm^2 anti-RE Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

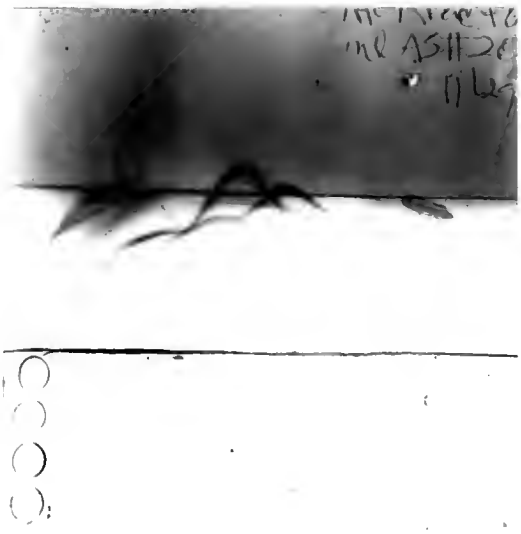
°60 ul ^{14}C-glucosamine labeled HEp-2 mock infected IEP antigen and 12 ul/cm^2 anti-RE Ig

*RMV range of complex of 2 or 3 peaks

FIGURE 25

Crossed immunoelectrophoretic analysis of the pattern of glycosylated antigens of the McKrae strain. Cultures of HEp-2 cells were infected with McKrae or mock infected and grown in medium supplemented with ^{14}C-glucosamine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 6 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the McKrae strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 90 ul of McKrae antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C.

A



B

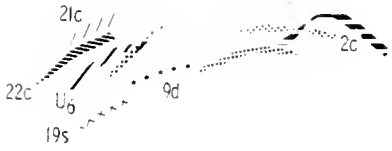
C

D

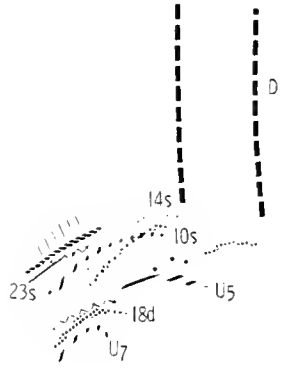
FIGURE 26

Tracing of Figure 25 of the pattern of glycosylated antigens of the McKrae strain. Reference gel contained 6 ul/cm^2 anti-McKrae Ig. (A) Coomassie brilliant blue staining of 90 ul of McKrae antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,H are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen; U is unknown antigen.

A



B



C

D

TABLE 15. GLYCOSYLATED ANTIGEN PATTERN OF McKRAE

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-McKRAE RMV RANGE† CBB§	HEp-2-McKRAE RMV RANGE† FLUOROGRAPHY	HEp-2 MOCK INFECTED° RMV CBB§	HEp-2 MOCK INFECTED° RMV FLUOROGRAPHY	VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS
A	64-72		69-71*		-
B	53-58		57-59*		-
2c	54-58				-
C	51-56	52-61	53		-
D		32			-
9d	33-37	36-41			+
10s	27-35	31-37			+
14s	28-35	32-35			+
H	23-28		23		-
18d		14-17			+
19s	16-21	16-22			+
21c	16-18	16-18			+
22c	15-18	16-18			+
23s		13			+
U_5		32-34			+
U_6	21-24	18-28			+
U_7		14-17			+
					11/17

•A,B,C,D,H are nonspecific peaks; c is common antigen, d is distinguishing antigen, s is shared antigen, U is unknown antigen

¶60 or 90 ul ^{14}C-glucosamine labeled HEp-2 infected with McKrae IEP antigen and 6 or 8 ul/cm^2 anti-McKrae Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

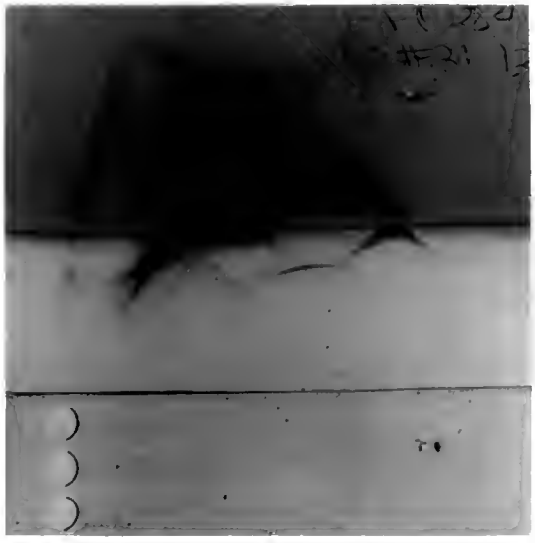
°60 ul ^{14}C-glucosamine labeled HEp-2 mock infected IEP antigen and 6 ul/cm^2 anti-McKrae Ig

*RMV range of complex of 2 or 3 peaks

FIGURE 27

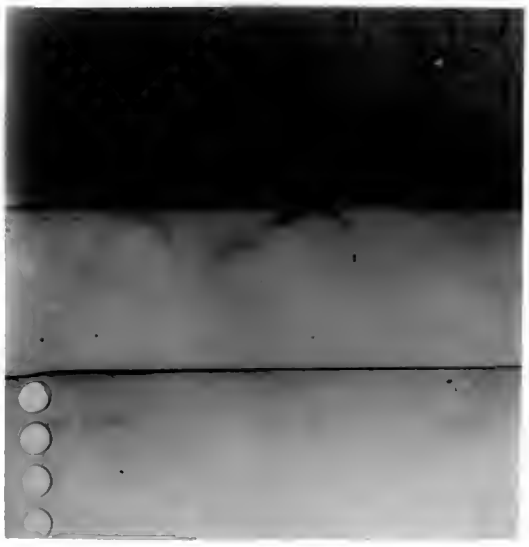
Crossed immunoelectrophoretic analysis of the pattern of glycosylated antigens of the F strain. Cultures of HEp-2 cells were infected with F or mock infected and grown in medium supplemented with ^{14}C-glucosamine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin (Ig) rich fraction of antiserum to the F strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 60 ul of F antigen and 10 ul/cm^2 anti-F Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen and 12 ul/cm^2 anti-F Ig. (D) Fluorography of C.

A



B

C



D

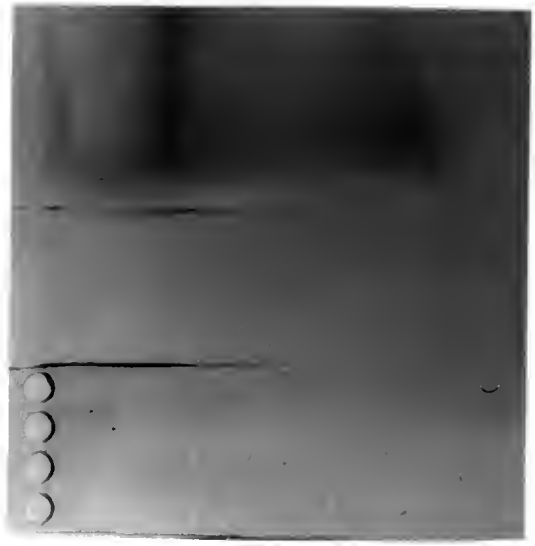
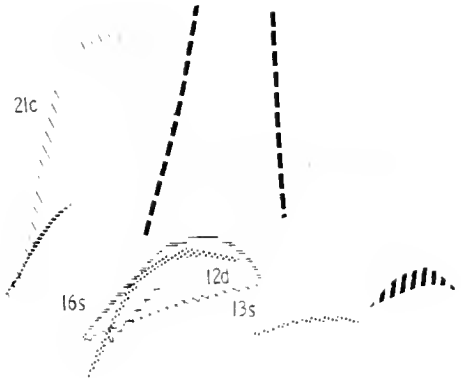


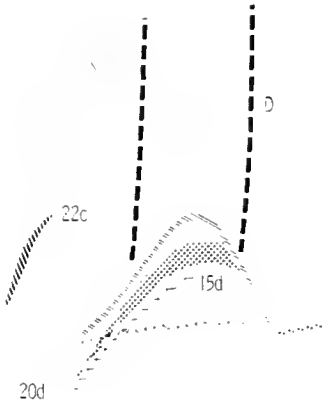
FIGURE 28

Tracing of Figure 27 of the pattern of glycosylated antigens of the F strain. The reference gel contained anti-F Ig. (A) Coomassie brilliant blue staining of 60 ul of F antigen and 10 ul/cm^2 anti-F Ig. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen and 12 ul/cm^2 anti-F Ig. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D, F,G,H are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen. Immunoprecipitation peak 22c was not resolved in the gel in Figure 27A but was detected in at least two other gels of the reference pattern of the F strain.

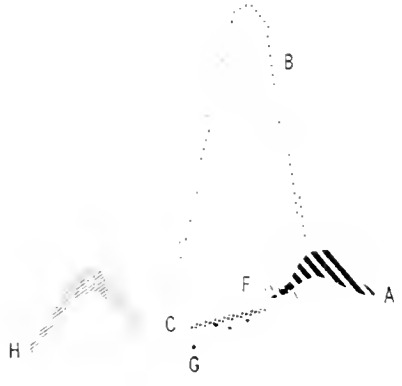
A



B



C



D

TABLE 16. GLYCOSYLATED ANTIGEN PATTERN OF F

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-F RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2 MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS
A	61-67		68-69*		-
F			64		-
B	52-54		53		-
C	48-46	48-50	53		-
G			51		-
D	29	27-29			-
12d	17-25	17-25			+
13s	23-32	33-36			+
H	24-25		22		-
15d	20-25	20-27			+
16s	25-27	25-27			+
20d	13	11-13			+
21c	11-14	13-14			+
22c	7	6-7			+
					7/14

•A,B,C,D,F,G,H are nonspecific peaks; d is distinguishing antigen, s is shared antigen, c is common antigen

¶45 or 60 ul ^{14}C-glucosamine labeled HEp-2 infected with F IEP antigen and 10 or 12 ul/cm^2 anti-F Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

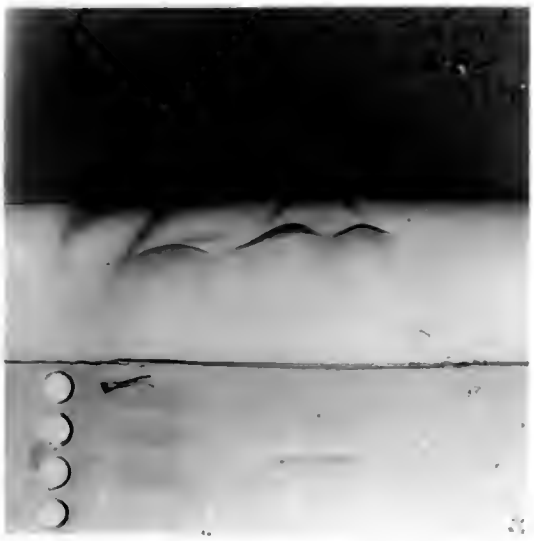
°60 ul ^{14}C-glucosamine labeled HEp-2 mock infected IEP antigen and 12 ul/cm^2 anti-F Ig

*RMV range of complex of 2 or 3 peaks

FIGURE 29

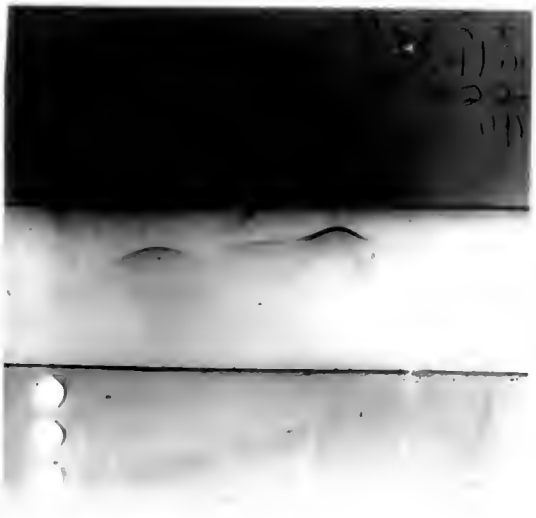
Crossed immunoelectrophoretic analysis of the pattern of glycosylated antigens of the CGA-3 strain. Cultures of HEp-2 cells were infected with CGA-3 or mock infected and grown in medium supplemented with ^{14}C-glucosamine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 12 ul/cm^2 of the immunoglobulin (Ig) rich fraction of antiserum to the CGA-3 strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described in Materials and Methods. (A) Coomassie brilliant blue staining of 60 ul of CGA-3 antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C.

A



B

C

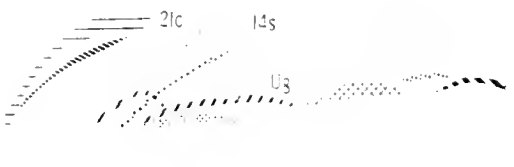


D

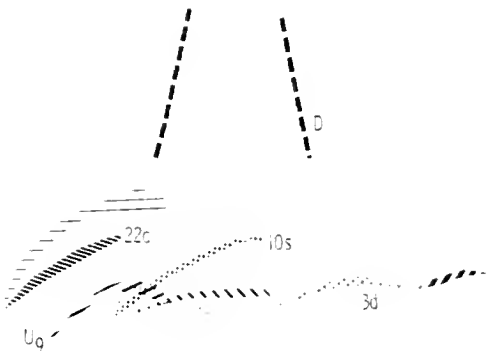
FIGURE 30

Tracing of Figure 29 of the pattern of glycosylated antigens of the CGA-3 strain. Reference gel contained 12 ul/cm^2 anti-CGA-3 Ig. (A) Coomassie brilliant blue staining of 60 ul of CGA-3 antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 60 ul of HEp-2 mock infected antigen. (D) Fluorography of C. Immunoprecipitation peaks labeled: A,B,C,D,E are nonspecific peaks; c is common antigen, s is shared antigen, d is distinguishing antigen; U is unknown antigen.

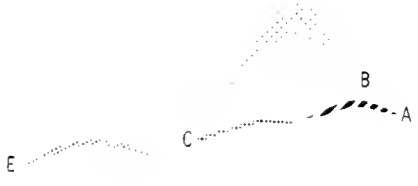
A



B



C



D

TABLE 17. GLYCOSYLATED ANTIGEN PATTERN OF CGA-3

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-CGA-3 RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2 MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS
A	64-68	69	68*		-
B	53-58		57-58*		-
C	61		54		-
3d	49-52	48-53			+
D		27-31			-
10s	25-33	26-34			+
14s	25-33	28-32			+
E	23-26*	23-26	22		-
21c	15-22	16-20			+
22c	14-16	12-17			+
U_8	30-33	30-34			+
U_9	16-18	14-16			+

7/12

•A,B,C,D,E are nonspecific peaks; d is distinguishing antigen, s is shared antigen, c is common antigen, U is unknown antigen

¶60 ul ^{14}C-glucosamine labeled HEp-2 infected with CGA-3 IEP antigen and 8, 12 or 15 ul/cm^2 anti-CGA-3 Ig

†Relative migration velocity of immunoprecipitation peaks of 3 gels

§Coomassie brilliant blue

°60 ul ^{14}C-glucosamine labeled HEp-2 mock infected IEP antigen and 12 ul/cm^2 anti-CGA-3 Ig

*RMV range of complex of 2 or 3 peaks

TABLE 18. SUMMARY OF VIRUS-SPECIFIC GLYCOSYLATED ANTIGENS FROM FLUOROGRAPHY OF ^{14}C-GLUCOSAMINE LABELED STRAINS

IMMUNOPPT. PEAK DESIGNATION	SHEALY	RE	McKRAE	F	CGA-3
3d*					X
9d			X		
10s§	X		X		X
12d				X	
13s	X	X		X	
14s	X	X	X		X
15d				X	
16s	X			X	
18d			X		
19s	X	X	X		
20d				X	
21c¶	X	X	X	X	X
22c	X	X	X	X	X
23s	X		X		
U_1†	X				
U_2		X			
U_3		X			
U_4		X			
U_5			X		
U_6			X		
U_7			X		
U_8					X
U_9					X
Virus-specific Antigens	9	8	11	7	7

*d is distinguishing antigen
§s is shared antigen
¶c is common antigen
†U_{1-9} are unknown immunoprecipitation peaks which do not match peaks of reference patterns or HEp-2 mock infected patterns

DISCUSSION

In the past there has been much emphasis on the differences between the two serotypes, HSV-1 and HSV-2, particularly in relation to their site of location in the human body (15,28,33,34,43,53). Presently the similarities and differences among various strains of HSV are being studied (28,34), and there is evidence associating strain characteristics with particular ocular disease states (8,9). The work presented here demonstrates a correlation of the antigenic composition of five strains of HSV type 1 and the characteristic eye disease that they cause.

Source of Antisera

The antigenic composition of these strains was determined by cIEP. Since cIEP is a serological technique in which the detection of antigens is dependent on the available precipitating antibodies, the source and properties of the antisera are important. The antisera used in this study were obtained from rabbits immunized i.m. and boosted by infection in the eyes with RK-13 cells that had been infected with each HSV strain passaged three times without FCS.

Two points are important. First, in order to obtain antiserum to the antigens which are important in ocular disease, the rabbits were boosted by infection in the eyes and thus exposed to the virus at the site of natural infection. Some of these eyes did show the characteristic ocular disease pattern. Therefore, the rabbit should have produced antibodies to antigens available both from immunization

and from the ocular disease. Other laboratories have shown differences in immune response to HSV antigens by immunization and by infection. For example, Vestergaard et al. (73) has demonstrated that infected rabbits (that received both the cells disrupted by sonication in water and the released infectious particles) developed precipitating antibodies against a larger number of HSV antigens than immunized rabbits (that received cells solubilized in Triton X-100 without infectious virus).

Second, initial studies indicated that artifacts caused by FCS needed to be eliminated (data not shown). To avoid this, medium added to infected cell cultures initially did not contain FCS. However, cell cultures grew better with FCS and resolution on cIEP was better with these antigens. Thus, cell cultures to be used for antigen preparation were washed extensively with PBS before infection and the medium, which was added after virus adsorption, contained 1% FCS.

To avoid raising antibodies to HEp-2 cells, the host cell for the antigen preparation, rabbits were immunized with RK-13 infected cells. With these antisera, only five nonspecific antigens (A,B,C,D,E) were resolved on the HEp-2 mock infected cells with the five antisera and on the reference patterns of the five strains. These antigens were probably common host cell proteins found in both HEp-2 and RK-13 cell cultures or medium components. Three additional antigens (F,G,H) resolved by CBB staining of ^{14}C-glucosamine labeled HEp-2 mock infected cells and on the glycosylated antigen pattern of the five strains were presumably common host cell proteins. Therefore, the majority of antigens resolved on the reference patterns of the five

strains were not identifiable on the HEp-2 mock infected pattern and were virus-specific antigens. The immunoprecipitation peaks which matched with peaks on the HEp-2 mock infected cells were classified as nonspecific.

Unknown Antigens

In the experiments demonstrating cross-reacting antigens and glycosylated antigens, the immunoprecipitation peaks that did not match with the 23 virus-specific antigens of the five reference patterns or the eight nonspecific antigens of the HEp-2 mock infected pattern were classified as unknown. There were 13 unknown antigens in the 20 experimental gels which demonstrated the cross-reacting antigens. Since the same antigen preparations and antisera were used previously in the reference patterns, these 13 antigens may have been antigens of the reference patterns but the antigen/antibody ratio altered the shape or relative migration of the peaks in the second dimensional gel so that they could not be matched exactly. Another possibility is that these 13 peaks were cross-reacting moieties of one of the antigens of the reference pattern and appeared different; therefore, they could not be matched exactly by shape, location, or RMV.

There were nine unknown antigens (U_{1-9}) resolved in the glycosylated antigen pattern. Vestergaard and Norrild, in their study of HSV antigens using cIEP, have demonstrated that the quantity of HSV antigens varies in different preparations since it is difficult to standardize cell culture conditions (72) and have shown that some antigens are not consistently resolved (48,50). The temporal events of antigen synthesis are significant in that some antigens are expressed

early and some late (16). Also, the major glycoproteins in the infected cell are glycosylated in two stages, resulting in partially glycosylated intermediates and fully glycosylated products (64). Since another antigen preparation of each strain was used in determining the glycosylated antigens, these nine unknown antigens were presumably glycosylated intermediates or early or late antigens which were found in different quantities in the ^{14}C-amino acid labeled antigen preparations.

Similarities among These Five Strains

The similarities and differences among the five strains of HSV were illustrated by the three classifications of the 23 virus-specific antigens, by the reference patterns and by the cross-reacting antigens. The data show that there is much cross-reactivity among these strains and that differences among very similar strains are being compared. This interpretation is supported by the report of Pereira *et al.* (51). Variation in the electrophoretic mobility of five structural polypeptides of 53 strains of HSV-1 isolated from four countries was shown by SDS-PAGE. When the distribution of the strains was compiled according to the site of isolation on the human body, the majority (five of eight) of the corneal isolates were found in the same group. The distribution indicated that the biochemical properties of the strain may be related to the site of its localization on the human body.

One interesting point illustrated by the data of the experiments which demonstrated cross-reacting antigens was that each antiserum resolved antigens of a heterologous strain but did not resolve the same

antigens in the reference pattern of the control strain. The example of shared antigen 7s was described in the Results section, above. This phenomenon is explained by the following hypothesis. Some viral antigens have cross-reacting moieties that exist in varying amounts and spatial configurations within the strains. In some strains, the amount and configuration of a moiety is such that it is resolved as an immunoprecipitation peak, whereas the same moiety in another strain is not resolved as a peak. This hypothesis is supported by Hampar and Martos (16) who propose that common or cross-reacting antigens may be present in varying amounts and spatial configurations on the surface of the virion. This explanation is also pertinent to the definition of distinguishing antigen and applies to all of these antigens except 1d and 20d: distinguishing antigens were found in the reference pattern of one strain with homologous antiserum or in the same strain with heterologous antisera or if present in a different strain, were resolved only by the original homologous antiserum.

Glycosylated Antigens

The glycosylated antigen pattern emphasizes the significance of the glycoproteins in that the majority, 14 of 23, of the virus-specific antigens were glycosylated. Antigens from each of the three classifications were glycosylated: two of three common, six of 10 shared and six of 10 distingushing antigens. Glycoproteins of the HSV virion are important in adsorption and penetration of the host cell, in eliciting neutralizing antibodies (67,75,76,78), in the social behavior of HSV infected cells (20,21,60) and presumably in the disease process (72,76).

Correlation of the Antigens of These Five Strains with Previous Studies

The number of virus-specific antigens, 8 to 13, of the reference patterns of the five strains of HSV-1 resolved in this study agrees fairly well with previous studies (48,50,71,72,73,76,77). Using the same procedure of cIEP in agarose gels, Vestergaard and Norrild and associates have shown seven antigens in various strains of HSV-1: 3, 3A, 5, 6, 7, 8, 11. Four of these antigens are detected consistently and have been studied extensively and characterized; Ag 6, 8, and 11 are membrane-bound, glycosylated antigens and Ag 3 is not glycosylated.

Antigens corresponding to Ag3, 6, 8, and 11 are identified in the reference patterns of the five strains of HSV type 1 of this study (Norrild, personal communication; Vestergaard, personal communication). Based on RMV, shape, and location of the immunoprecipitation peaks from various studies using cIEP in agarose gels, Ag3 probably corresponds to 2c, Ag8 to 21c and 22c and 23s, Ag11 to 13s and 16s, Ag6 to 6s or 10s and 14s. Additional studies using monoprecipitin antisera to Ag8 and 11 (gifts from Dr. Bodil Norrild) will be done to confirm these corresponding antigens. The glycosylated antigen patterns of the five strains of this study also show that these antigens are comparable to those described by Norrild and Vestergaard. As reviewed above, Ag3 is not glycosylated, and 2c described in this study is also not glycosylated. Norrild and Vestergaard have shown that Ag6, 8, and 11 are membrane-bound, glycosylated antigens; and all of the antigens of this study which possibly correspond to these previously described

antigens are glycosylated, except 6s which is not found on the glycosylated antigen patterns.

Relationship of Antigens with Glycoproteins

There is a relationship between antigens Ag6, 8 and 11 and the major glycoproteins VP8(C_2), VP7(B_2), VP8.5(A), and VP18(D_2). Antigen 6 is composed of VP8(C_2), Ag8 of VP18(D_2), and Ag11 of VP8.5(A) plus VP7(B_2) (48,49). These antigenically distinct glycoproteins are in the infected cell membrane, and the fully glycosylated forms correspond to the structural virion glycoproteins VP8, VP7, VP8.5 and VP18 (19,64). Cohen and associates (10) studied the CP-1 antigen, which is a type common glycosylated antigen that elicits the production of neutralizing antibodies. In correlating their results with other laboratories, they stated that CP-1 is comparable to glycoprotein VP18(D_2) of Spear et al. (64,65), VP17, 18, 19E of Heine et al. (19), Ag8 of Norrild and Vestergaard (49), and 8/9 of Powell et al. (52). Norrild and Vestergaard (49) also correlate Ag6 with Courtney and Powell's major virion glycoprotein (12).

The relationship of the antigens with the glycoproteins is important in determining the functions of the various glycoproteins, increasing our understanding of the interaction of the virus with the host cell in vitro, and possibly finding antigenic determinants involved in virulence. The importance of this relationship is illustrated by glycoprotein VP7(B_2) and Ag11 which is composed of glycoproteins VP8.5(A) and VP7(B_2) (48,49). Glycoprotein VP7(B_2) is required for infectivity, it is necessary for penetration, and it also plays a significant role in HSV-induced cell fusion. Both

glycoprotein VP7(B_2) and Agll are highly immunogenic in rabbits and in humans. Glycoprotein VP7(B_2) elicits neutralizing antibody in immunized rabbits (67), Agll elicits the highest neutralizing titer in rabbits (75,76), antibodies to this antigen are found in highest titer in human sera (72), and monoprecipitin antiserum to Agll mediates immunocytolysis (48).

Ocular Disease Caused by These Five Strains

Centifanto and associates (8,9) are presently correlating differences of HSV laboratory strains and clinical isolates with the varying forms of eye disease they produce. She has compared the biological properties in vitro and polypeptide composition of the five strains used in this study with the pattern, severity, and virulence of ocular disease (8,9). The disease pattern of each of the five strains was followed after inoculation into the corneas of New Zealand white rabbits (79). Table 19 demonstrates differences among this homogeneous group of viruses as shown by variation in the kind, severity, and percentage of eyes involved in disease. The kind of disease varied from epithelial only (produced by all five strains) to a combination of epithelial and stromal (produced by four strains). The severity of epithelial disease ranged from mild dendritic ulcers to geographic defects, which involved more of the eye. In stromal keratitis, disciform edema can progress into stromal necrosis with total corneal vascularization, which is the most severe disease state. Also, the percentage of eyes involved varied from 0 to 100 percent.

Shealy and RE strains cause the most severe epithelial and stromal disease, and the infectious dose 50 (ID_{50}) illustrates the

virulence (8); the ID_{50} for the Shealy strain is less than 3 and for the RE strain is 2.75. The McKrae strain causes moderate ocular disease and has an ID_{50} of 4.5. Note that the McKrae strain causes death by encephalitis in the majority of rabbits infected with this strain. Strain F causes moderate disease and has an ID_{50} of 3.78. CGA-3 causes only mild epithelial disease and has an ID_{50} of 5.18. The ID_{50}'s for the virulent strains, Shealy and RE, are much higher than those for CGA-3, which causes mild disease. There is a relationship between titer, incidence and kind of disease; a high titer does not necessarily cause severe disease and the disease pattern remains true independent of titer. A low titer (10^3 PFU) of a virulent virus that causes stromal disease (e.g., Shealy) continues to produce severe stromal disease. A high titer (10^6 PFU) of a nonvirulent virus that causes epithelial disease (e.g., CGA-3) does not produce severe disease and does not produce stromal disease (8).

Correlation of the Antigenic Composition with the Ocular Disease Pattern

The antigenic composition of these five strains correlates with the ocular disease they produce, as shown in Table 20. Both the kind and severity of disease is associated with the number of virus-specific antigens and the number of shared antigens. As the severity of disease increases, the number of virus-specific antigens increases. For example, Shealy and RE strains, which cause the highest percentage of and the most severe degree of stromal disease, have the largest number of viral specific antigens, 13, compared with CGA-3 strain, which causes mild disease and has only eight virus-specific antigens. These

data indicate that antigenic load may be important in the severity of the disease and, in particular, in stromal disease, which has an immunopathological basis involving host response to viral antigen.

Similarities in the antigenic composition of these five strains are related to the kind of disease they produce. The four strains that cause stromal disease (Shealy, RE, McKrae, and F) share more antigens with each other than with CGA-3, which causes only mild epithelial disease (Tables 6,20). These similarites were used in correlating antigens that may have a role in the two different disease patterns. Since all five strains cause epithelial disease, the three common antigens (2c, 21c and 22c) associated with all five strains and the three antigens (10s, 14s and 23s) shared by CGA-3 and at least two of the other four strains are associated with epithelial disease. Note that all of these six antigens associated with epithelial disease are glycosylated except 2c. In the four strains that cause stromal disease, seven antigens (5s, 6s, 7s, 8s, 13s, 16s 19s) are shared by at least two of the strains. Three (13s, 16s, 19s) of these seven antigens associated with stromal disease are glycosylated. These data indicate a correlation between the antigenic composition of the HSV strain and the ocular disease. However, many more strains need to be investigated to confirm this correlation, and these data do not exclude the possibility that other antigens may be involved in the disease process. Perhaps antigens that were not detected on cIEP are important.

There is variation in the kind, severity, and virulence of disease produced by different strains of HSV, and these differences are well

illustrated by the epithelial and stromal disease patterns (8,79). This variation in disease is dependent upon host factors and strain characteristics of the infecting virus. Recently, investigators have been studying strain differences in relation to eye disease. The technique presented in this study can be used to characterize additional laboratory strains as well as clinical isolates. The correlation of the antigenic composition of many strains of HSV with the characteristic eye disease they cause in rabbits and with other biological and biochemical properties may aid in our understanding of the pathogenesis of HSV in human disease.

TABLE 19. INCIDENCE OF EPITHELIAL AND STROMAL DISEASE

VIRUS STRAIN	PERCENTAGE OF EYES WITH EPITHELIAL DISEASE		PERCENTAGE OF EYES WITH STROMAL DISEASE	
	DENDRITIC DEFECTS	GEOGRAPHIC DEFECTS	STROMAL DISEASE	STROMAL NECROSIS WITH TOTAL CORNEAL VASCULARIZATION
SHEALY	100	60	80	15
RE	95	50	70	10
McKRAE†	80	30	60	0
F	75	0	50	0
CGA-3	45	0	0	0

*Classification and incidence of ocular disease was determined in the corneas of New Zealand white rabbits as described by Wander et al. (79).

†Death from encephalitis in the majority of animals infected with the McKrae strain

TABLE 20. CORRELATION OF VIRUS-SPECIFIC ANTIGENS WITH SEVERITY OF OCULAR DISEASE

VIRUS STRAIN	SEVERITY OF DISEASE*	NO. OF ANTIGENS OF REFERENCE PATTERNS	NO. OF SHARED ANTIGENS
SHEALY	SEVERE	13	10
RE	SEVERE	13	7
McKRAE	MODERATE	12	7
F	MODERATE	11	5
CGA-3	MILD	8	3

*The severity of disease was determined in corneas of New Zealand white rabbits as described by Centifanto et al. (8).

APPENDIX A

TECHNICAL ASPECTS OF CROSSED IMMUNOELECTROPHORESIS

INTRODUCTION

In order to work out the technical aspects of the crossed immunoelectrophoresis technique, antigen preparations of five strains of HSV grown in medium without radioactively labeled components were subjected to electrophoresis using this technique.

MATERIALS AND METHODS

Virus Strains and Cell Culture

The same strains and procedures for obtaining stock virus were followed as described in the Materials and Methods in the main text.

Preparation of Antigen

Roller bottles of HEp-2 cells were infected with each of the five viruses following the procedure for infection as described in the Materials and Methods in the main text for growing virus stocks of very high titers. Cultures were harvested at 24 to 48 h when the majority of cells were infected (4+ cytopathic effect) and the same procedure for the preparation of antigen was followed as described in the Materials and Methods in the main text. Cultures of HEp-2 cells were mock infected and treated similarly. The proteins for each of the antigen preparations were determined by adapting the Lowry protein assay (36). The protein concentrations in milligram/milliliter (mg/ml)

were Shealy, 6.5; RE, 7.8; McKrae, 5.5; F, 6.5; CGA-3, 4.8; and HEp-2 mock infected cells, 8.0.

Preparation of Antisera

The same antisera used in the main text were also used in these experiments.

Crossed Immunoelectrophoresis

The same procedure was followed as described in the Materials and Methods in the main text with the following exceptions. The antigen and phenol red were subjected to electrophoresis from the negative to the positive pole at high voltage for 90 min and the immunoprecipitation peaks were visualized only by Coomassie brilliant blue staining.

RESULTS

Different volumes of antigen and antibody were used to determine the best resolution which was based on separation and density of the immunoprecipitation peaks. Reproducible patterns were obtained with each strain using different antigen/antibody ratios. Changes in this ratio altered both the separation and density of the peaks (Figure 31). As the volume of the McKrae antigen increased, faint immunoprecipitation peaks were more defined, peaks were separated more and thus the number of peaks increased. Note A and B of Figure 31. As the volume of antiserum to the McKrae strain increased, the immunoprecipitation peaks were more dense and less separated and did not migrate as far in the second dimension. Note A, B, and C of Figure 31. Similar results were obtained with the other four strains (data not shown).

Profiles of crossed immunoelectrophoresis of these unlabeled antigen preparations were compared with the reference patterns of the radioactively labeled strains. Profiles of two of the strains, McKrae and CGA-3, were compatible. Compare A of Figure 5 with D of Figure 31 showing the McKrae patterns. The profiles of the other three strains were different (data not shown); however, additional ratios of unlabeled antigen/antibody needed to be examined.

DISCUSSION

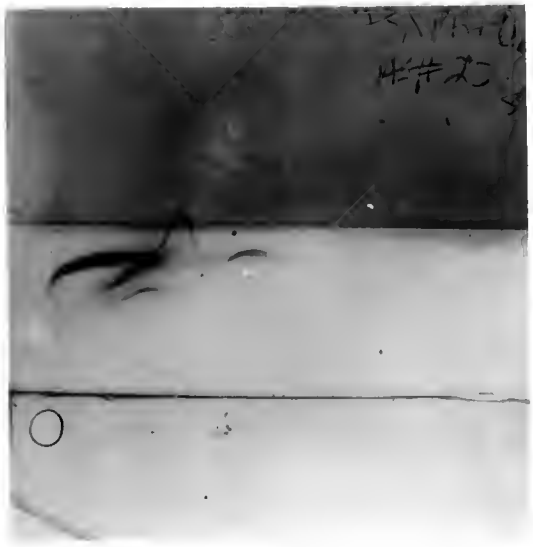
Obtaining both reproducible patterns with different antigen/antibody ratios and profiles with unlabeled antigen preparations that were comparable to those of the radioactively labeled antigens supported the validity of the results presented in this study. Since antigens presumably are not present in the same quantity or form in different preparations because parameters of cell cultures differ and are difficult to standardize (72), differences in the profiles of labeled and unlabeled antigens are expected and were found with three strains.

By using antigen preparations of the five strains of HSV type 1 that were not radioactively labeled, various parameters of the crossed immunoelectrophoresis technique were explored until the conditions resulting in the best resolution were found. These conditions were then used in determining the reference patterns with the radioactively labeled strains.

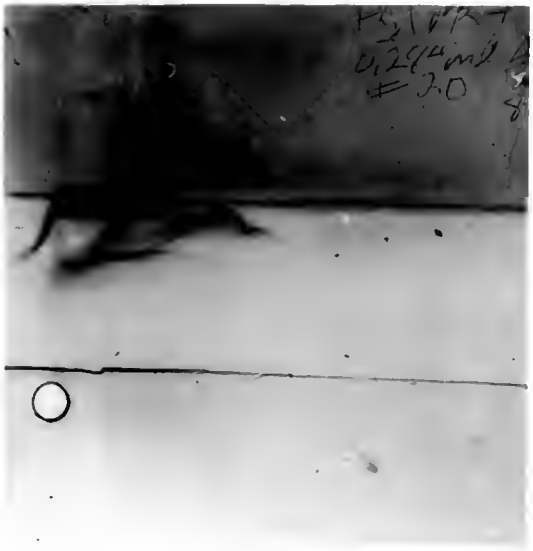
FIGURE 31

Crossed immunoelectrophoretic analysis of the McKrae antigen. Cultures of HEp-2 cells were infected with McKrae. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained the immunoglobulin rich fraction of antiserum to the McKrae strain grown in rabbit kidney-13 cells without FCS. Crossed immunoelectrophoresis was performed as described under Materials and Methods. Coomassie brilliant blue staining of (A) 5 ul antigen, 10 ul/cm^2 antiserum. (B) 15 ul antigen, 10 ul/cm^2 antiserum. (C) 15 ul antigen, 8 ul/cm^2 antiserum. (D) 15 ul antigen, 6 ul/cm^2 antiserum.

A

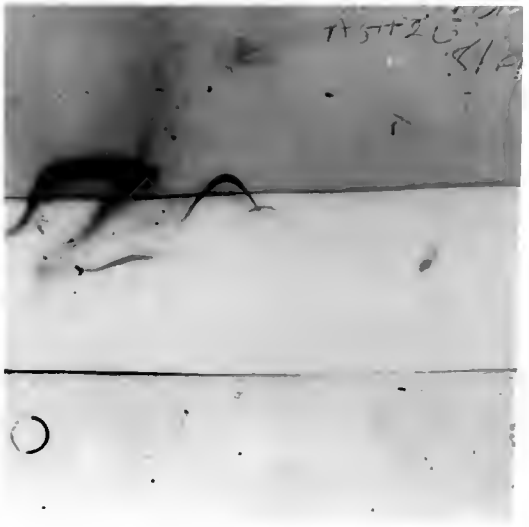


B



C

D



APPENDIX B

ANTIGENIC COMPOSITION OF ONE STRAIN OF HERPES SIMPLEX VIRUS TYPE TWO

INTRODUCTION

The antigenic composition of one strain of HSV type 2 was analyzed using crossed immunoelectrophoresis and was compared with the profiles of the five strains of HSV type 1.

MATERIALS AND METHODS

Virus Strain and Cell Culture

The Hicks strain of HSV type 2 was originally obtained from Dr. Andre Nahmias (Emory University). The procedure described in the Materials and Methods in the main text was used to obtain stock virus.

Preparation of Antigen

A roller bottle of HEp-2 cells was infected with the Hicks strain following the procedure described in the Materials and Methods in the main text for growing virus stocks of very high titers. Cultures were harvested at 24 or 48 h when the majority of cells were infected (4+ cytopathic effect). The same procedure was followed for the preparation of Hicks antigen for crossed immunoelectrophoresis as previously described for HSV type 1 strains. The protein concentration of this antigen was determined by an adaptation of the Lowry protein assay (36) to be 5 mg/ml. To obtain infected cells with radioactively

labeled amino acids, HEp-2 cells were infected with 27 PFU/cell of the Hicks strain (grown in a roller bottle). The cpm/0.1 ml of this antigen preparation was 183,632.

Preparation of Antiserum

Three New Zealand white rabbits were immunized with Hicks strain grown in human embryonic kidney cells. Two ml was injected intraperitoneally and 1 ml i.m. weekly for three weeks. The rabbits were bled eight days later. The bleedings from the three rabbits were pooled and the immunoglobulin fraction was obtained following the procedure for ammonium sulfate precipitation described in the Materials and Methods in the main text. Since the virus used for the immunogen had been grown in medium containing FCS, the immunoglobulin fraction was absorbed with an equal volume of FCS by gently mixing and absorbing overnight at 4°C. The mixture was spun at 100,000 x g for 1 h in the ultracentrifuge and the supernatant was reserved, tested in Ouchterlony plates, and used in crossed immunoelectrophoresis.

Crossed Immunoelectrophoresis

The procedure was followed as described in the Materials and Methods in the main text.

Microneutralization

The procedure for microneutralization described in the Materials and Methods in the main text was followed. The virus dilution contained 4.4×10^3 PFU/ml.

RESULTS

Antigenic Composition of One Strain of HSV Type Two

Five antigens designated 1 through 5 based on RMV were detected in the Hicks strain. Note A and B of Figure 32. The RMV of each antigen was calculated (Table 21). Since there were no peaks detected on the HEp-2 mock infected cells on either CBB staining or fluorography (C and D, Figure 32), all five antigens were designated virus-specific. There was a partial peak detected to the left of antigen 1; however, the cathodic leg of this peak was never resolved in either CBB staining or fluorography and thus was omitted.

Microneutralization Assay

The microneutralization titer of anti-Hicks Ig absorbed 1:1 with FCS was -3.166.

DISCUSSION

The number of virus-specific antigens of HSV-2 resolved in this study agrees fairly well with previous studies using the same procedure of cIEP in agarose gel. Vestergaard and Norrild and associates (71,72, 75,76; Norrild, personal communication) have identified eight antigens of various strains of HSV-2: 1, 2, 3, 4, 8, 9, 10, 11. Six of these antigens have been characterized: 4, 8, 9, and 11 are glycosylated and Ag1 and 3 are not glycosylated. The polypeptide composition and immunological significance of these antigens have been discussed in the Introduction of the main text. Based on the shape and location of the immunoprecipitation peaks, antigens designated 1, 2, 3, 4, 5 of the Hicks strain correspond to Ag1, 3, 4, 11, 8, respectively, in Vestergaard's and Norrild's studies.

By comparing RMV and location of the antigens of the one strain of HSV type 2 with the five strains of HSV type 1, antigens designated 2, 4, 5 of Hicks correspond with 2c, 19s, and 21c or 22c, respectively of the HSV type 1 strains, which correspond to Ag3, 11, and 8 of Norrild's and Vestergaard's studies. Since Ag3, 8 and 11 are type common antigens (71,72; Norrild, personal communication), these data support the comparison of antigens in type 1 and type 2 strains in this study. Additional experiments need to be done to confirm this hypothesis.

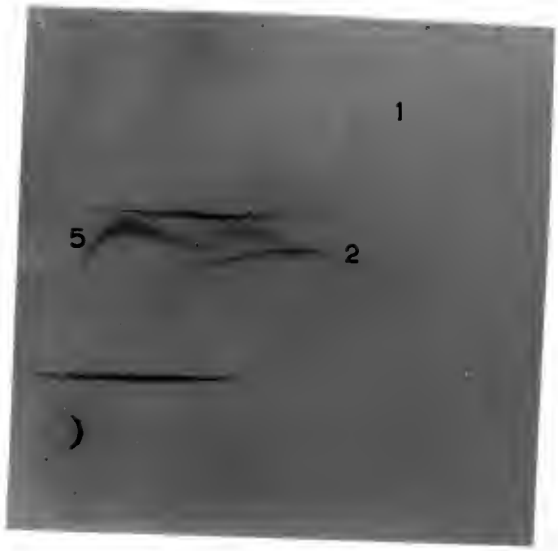
The severity and kind of ocular disease caused by the Hicks strain following inoculation in the corneas of New Zealand white rabbits was mild, with only 33 percent of eyes developing dendritic epithelial disease and 8.3 percent developing mild stromal keratitis (79). These data are in agreement with previous studies indicating that infections in the eye are caused primarily by HSV type 1 (53).

FIGURE 32

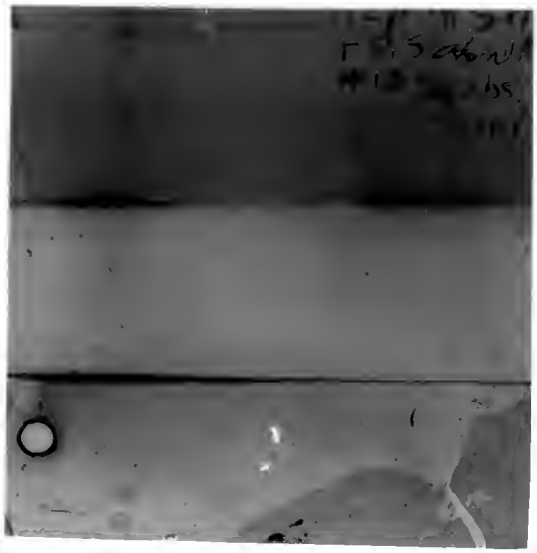
Crossed immunoelectrophoretic analysis of the reference pattern of the Hicks strain of HSV type 2. Cultures of HEp-2 cells were infected with Hicks or mock infected and grown in media without radioactive label or in media supplemented with ^{14}C-valine, -leucine, -isoleucine. Antigen was applied in the first dimensional electrophoresis, the intermediate gel contained agarose only, and the reference gel contained 10 ul/cm^2 of the immunoglobulin rich fraction of antiserum to the Hicks strain grown in human embryonic kidney cells and absorbed 1:1 with FCS. Crossed immunoelectrophoresis was performed as described under Materials and Methods. (A) Coomassie brilliant blue staining of 30 ul of unlabeled plus 15 ul of radioactively labeled Hicks antigen. (B) Fluorography of A. (C) Coomassie brilliant blue staining of 30 ul unlabeled plus 15 ul of radioactively labeled HEp-2 mock infected antigen. (D) Fluorography of C.

A

B



C



D

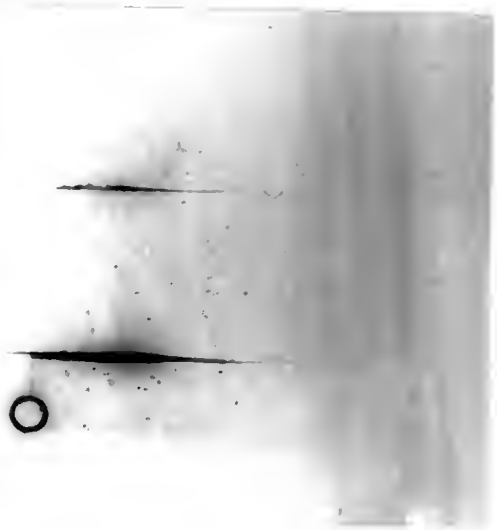


TABLE 21. REFERENCE PATTERN OF HICKS

IMMUNOPPT. PEAK DESIGNATION•	HEp-2-HICKS¶ RMV RANGE† CBB§	FLUOROGRAPHY	HEp-2-MOCK INFECTED° RMV CBB§	FLUOROGRAPHY	VIRUS-SPECIFIC
1	63-69	71-76			+
2*	45-51	49-59			+
3	36-38	39-41			+
4	18-22	20-21			+
5	11-16	10-12			+
					5/5

•Immunoprecipitation peak designation based on relative migration velocity

¶30 ul unlabeled plus 15 ul ^{14}C-val, -leu, -isl labeled HEp-2 infected with Hicks IEP antigen and 10 ul/cm^2 anti-Hicks Ig absorbed 1:1 with FCS

†Relative migration velocity of the immunoprecipitation peaks of 3 gels stained with CBB and fluorography of 2 gels. One gel contained 30 ul unlabeled antigen only

§Coomassie brilliant blue

°45 ul ^{14}C-val, -leu, -isl or 30 ul unlabeled plus 15 ul ^{14}C-val, -leu, -isl labeled HEp-2 mock infected IEP antigen and 10 ul/cm^2 anti-Hicks Ig absorbed 1:1 with FCS

*Greater range due to broad peak

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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